

STRUCTURAL STUDIES ON DNP
BINDING ANTIBODIES

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Trinity term, 1983

A thesis submitted in partial fulfilment of
the requirements of the degree of D. Phil.

ABSTRACT
Structural Studies on DNP Binding Antibodies

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This thesis is concerned with structural aspects of the recognition and effector functions of antibody molecules. The recognition process is investigated in the dinitrophenyl (DNP) binding mouse IgA produced by the myeloma MOPC 315. The studies on effector functions utilize a DNP binding mouse hybridoma IgG2a to examine the role of N-glycosylation in IgG.

The combining site of protein 315. The involvement of tyrosyl residues in the combining site of protein 315 was examined by preparing specifically nitrated NO₂-Tyr-33_H and NO₂-Tyr-34_L derivatives of the Fv fragment of this protein.² The ionizations² of these derivatives were studied in the presence and absence of various DNP-ligands. Perturbations to the nitrotyrosine ionizations were found to be caused by the side chains of certain of these ligands, allowing an indication of the distance of these tyrosines from the bound hapten. On examination of the compatibility of these data with the model of the combining site of protein 315 proposed by Dower *et al.* (1977) (Biochem. J. 165, 207-225) it was found that while the location of Tyr-33_H is consistent with this model, the position of Tyr-34_L is not.^H A remodelled combining site using the modified ring-current_L treatment of Perkins & Dwek (1980) (Biochemistry 19, 245-258) is presented. This allows a better rationalization of the nitration data and of previous experimental observations on protein 315.

The role of the conserved C_H2 domain oligosaccharide of IgG. This was examined by a functional_H comparison of native IgG with an aglycosylated IgG preparation. Aglycosylation was achieved by cell culture of the hybridoma cells in the presence of the glycosylation inhibitor tunicamycin. The conditions for preparation and purification of this aglycosyl IgG are described. Aglycosylated IgG is found to be correctly assembled as an H₂L₂ unit. It retains the antigen binding and Staphylococcal protein_A binding abilities of the native glycosylated molecule. Using an assay system designed specifically to overcome certain problems in comparing Clq binding to different preparations of IgG it was found that the aglycosylated preparation showed only slightly reduced affinity for Clq. In addition the aglycosylated IgG is able to activate bound Cl. The above results are consistent with the structure of the Fc region being only minimally altered in the absence of oligosaccharide. The structural integrity of the aglycosylated molecule may be compromised however, as its ability to bind to monocyte Fc receptor is significantly reduced. In addition the aglycosylated molecule becomes much more susceptible to proteolytic digestion. A computational model-building analysis of the quaternary structure of Fc allows an explanation of at least some of the effects of aglycosylation in terms of reduced conformational stability of the C_H2 domains.

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ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity.
BADE	N-(bromoacetyl)N'-(dinitrophenyl)-ethylenediamine.
BADL	α -bromoacetyl- ϵ ,N-DNP-L-lysine.
BBS	Borate buffered saline.
BSA	Bovine serum albumin.
BSS	Balanced salt solution.
BSS[-Ca,-Mg]	BSS without calcium and magnesium salts.
C	Constant.
CD	Circular dichroism.
C _x	Complement component x.
C _x ⁻	Complement component x activated.
DIBAB	α -N-bromoacetyl- γ -DNP-L-diaminobutyric acid N'-bromoacetyl hydrazide.
DNNS	2,4-dinitronaphthol-7-sulphonate.
DNP-	2,4-dinitrophenyl-.
δ	Chemical shift in ppm.
$\Delta\delta$	Change in chemical shift in ppm.
EDTA	Diaminoethane tetra-acetic acid.
E_{X}^{Y}	Extinction coefficient for concentration Y at wavelength = X nm. Pathlength = 1 cm.
FCS	Foetal calf serum.
H	Heavy chain.
IAA	Iodoacetamide.
Ig	Immunoglobulin.
K _d	Dissociation constant.
L	Light chain.
MNDB	m-nitrobenzene diazonium fluoroborate.
M _r	Relative molecular mass.

NMR	Nuclear magnetic resonance.
NOE	Nuclear Overhauser effect.
NPCGB	p-nitrophenyl p'-guanidino benzoate.
PBS	Phosphate buffered saline (150 mM NaCl / 20 mM phosphate, pH 7.2).
PBS / EDTA	PBS containing 2 mM EDTA.
PCA	Passive cutaneous anaphylaxis.
pH [*]	pH meter reading, uncorrected for the deuterium isotope effect.
ppm	Parts per million.
RMS	Root mean square.
SDS-PAGE	Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.
S.E.	Standard error.
SpA	Staphylococcal protein A.
TNM	Tetranitromethane.
TNP-	2,4,6-trinitrophenyl-.
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol.
V	Variable

NOMENCLATURE

Carbohydrates - Eur. J. Biochem. (1982) 126, 433-437

Numbering of amino acid side chains - J. Mol. Biol. (1970) 52, 1-17

Single letter notation for amino acids - Biochem. J. (1969) 113, 1-4

Immunoglobulins and fragments - Gally (1973)

Immunoglobulin tertiary structure - Beale & Feinstein (1976)

For chemical shift changes the following sign convention is used - upfield shifts are ones which result in a decreased chemical shift value.

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CHAPTER 1

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1.1 GENERAL INTRODUCTION

A characteristic of vertebrate organisms is the ability to distinguish self from non-self at a molecular level. The presence of non-self elicits the immune response, a reaction which neutralizes or destroys foreign molecules or cells (antigens). Fundamental to the recognition of foreign antigens are the immunoglobulins (antibodies). These are a heterogeneous mixture of multi-chained serum glycoproteins which constitute 25-30% of normal human blood plasma proteins.

Immunoglobulins may be divided both physically and functionally into three distinct regions. Two of these are identical (termed the Fab regions), the third has a different structure and is known as Fc. The Fab contains the antigen binding site which fulfills the primary role of recognition. However the binding of antigen by antibody is not in itself sufficient to cause destruction or modification of the antigen. Such processes are mediated via secondary systems which may involve both humoral and cellular components, and for which the Fc region provides the recognition site. Thus immunoglobulins provide the link between the extremely diverse nature of the different antigenic determinants presented by the environment, and the comparatively limited range of secondary systems available to deal with them. This dual functionality is a consequence of the unique structural features of the immunoglobulin molecule. This chapter provides an overview of these structural features, and also describes the functional characteristics of the immunoglobulins.

1.2 STRUCTURE OF IMMUNOGLOBULINS

1.2.1 General characteristics of immunoglobulins

Immunoglobulins (Ig) are composed of a 1:1 molar ratio of heavy chains (H, $M_r = 50,000 - 80,000$ daltons) and light chains (L, $M_r \sim 25,000$ daltons). These chains are associated by strong non-covalent interactions and usually (but not always) by disulphide bonds. The different types of heavy chain are designated α , δ , ϵ , γ , and μ ; light chains occur as two types, κ and λ . Immunoglobulins are subdivided further by:

Class Different classes have quite different physico-chemical / functional / serological characteristics. Both types of light chains are found paired with each of the various heavy chains. Since no known functional difference is associated with different light chains, the immunoglobulin class is determined by its heavy chain constant region to give classes A, D, E, G and M respectively from the different heavy chains listed above. Immunoglobulins of the same class also show serological homology, as the antigenic determinants of their constant regions are the same. They are therefore also known as isotypes.

Subclass Within a class of immunoglobulin there is often a range of slightly different heavy chains, which although sharing many of the same features are products of distinct genes. These give rise to different subclasses. The occurrence of subclasses is a common characteristic of IgG of many species, for example both humans, mice and rats have four IgG subclasses.

Allotype This is a difference within a subclass / class which is caused by allelic variation. The differences, which are detected serologically, may be caused by single or multiple amino acid

substitutions.

Idiotype Idiotypic variation is due to the unique feature of immunoglobulins of sequence variability which gives rise to the antigenic specificity of the molecule (see section 1.2.3). Idiotypic differences arise, therefore, due to the possession of different hypervariable sequences which confers different antigenic properties to individual immunoglobulin molecules. It should be noted that as with all immunoglobulin "-types" the definition is based on serological properties which unfortunately means that current usage is not quite as restricted as this (see Secher et al., 1979).

1.2.2 Immunoglobulin chain structure and the domain hypothesis

Despite the differences between the various immunoglobulin classes, they share a common feature. When their amino acid sequences are examined it is found that they all may be described in terms of a reasonably homologous ~ 110 amino acid repeating sequence containing an intra-chain disulphide (Hill et al., 1967). This led Edelman et al. (1969) to propose the "domain theory" - that immunoglobulins evolved by gene duplication, and that the homology regions will lead to a similar three-dimensional structure of a series of compact domains. It was further suggested that each of these domains would contribute to at least one active site. Although light chains always contain two domains the number of domains per heavy chain varies from four in IgA, IgD and IgG to five in IgM and IgE. It is found that the third domain of IgM and IgE has been truncated to give the "hinge" region of the other three classes. The hinge region is (usually) a relatively short extra-domain sequence characterized by a high proline content and containing one or more inter-chain disulphide bonds. Other extra-domain regions are found

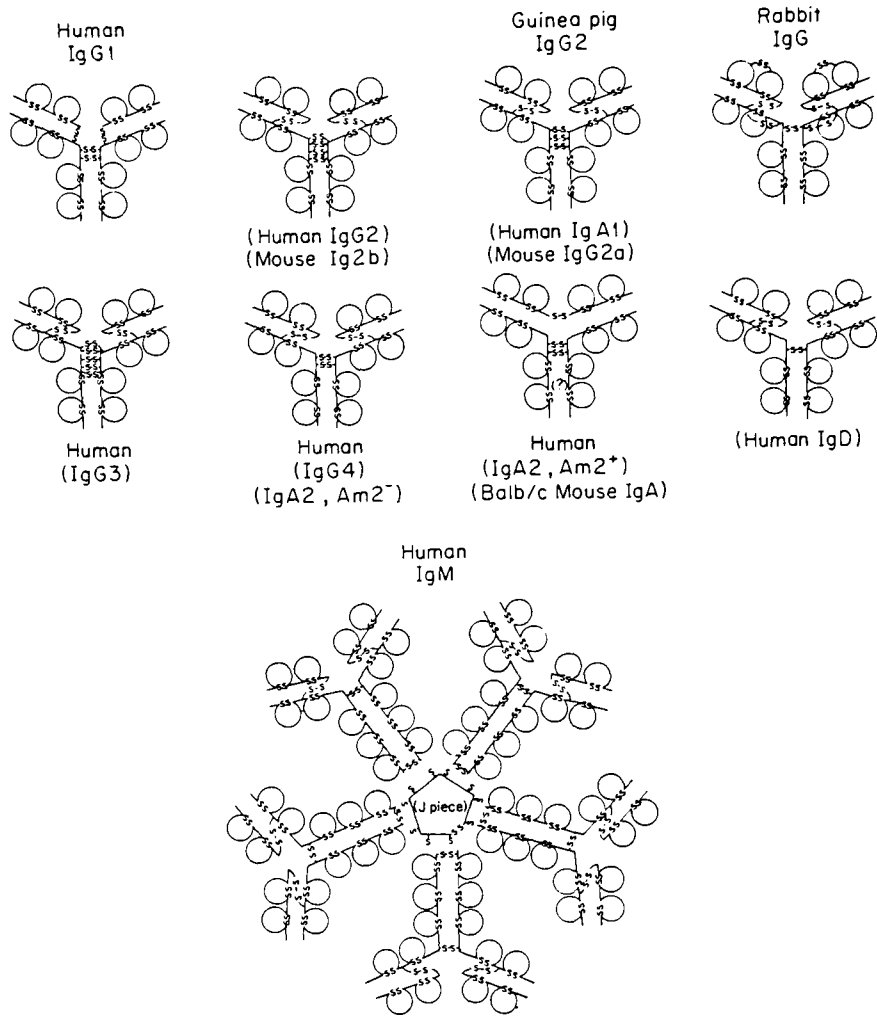


Figure 1.1 Chain structure and disulphide bonding pattern of immunoglobulins (from Gally, 1973).

in the C-terminal "tail piece" of IgA and IgM which (together with an additional protein, J-chain [Koshland, 1975]) is involved in the multimeric assembly of these classes. Membrane-bound IgG and IgM contain an alternative C-terminal sequence which is thought to be directly involved in membrane attachment (Alt et al., 1980; Early et al., 1980; Rogers et al., 1980; Singer et al., 1980; Owen & Kissonerghis, 1982).

The basic stoichiometry of immunoglobulins is $(H_2L_2)_n$, where $n = 1$ except for IgM where it is 5, and secretory IgA where it is 2. The chain structure and disulphide bonding pattern of various immunoglobulin classes and subclasses are shown in Fig. 1.1. This structure, in conjunction with the idea that the folding occurs to form discrete domains, rationalizes the characteristic proteolytic fragmentation which is undergone by immunoglobulins (Fig. 1.2) where large fragments may be formed which retain some but not all of the functionality of the parent molecule (Porter, 1959; Nisonoff et al., 1960; Inbar et al., 1972; Ellerson et al., 1976; Colomb & Porter, 1975).

1.2.3 Homogeneous antibodies

Immunoglobulins of any class isolated from serum display considerable heterogeneity in their V-region sequences (see section 1.2.4). This severely limits the usefulness of such polyclonal antibodies for detailed structural studies. Most work is therefore performed on monoclonal antibody preparations, which may be obtained in the quantities required for such studies in three ways.

1. Multiple myelomatosis is a lymphoproliferative disorder of the plasma

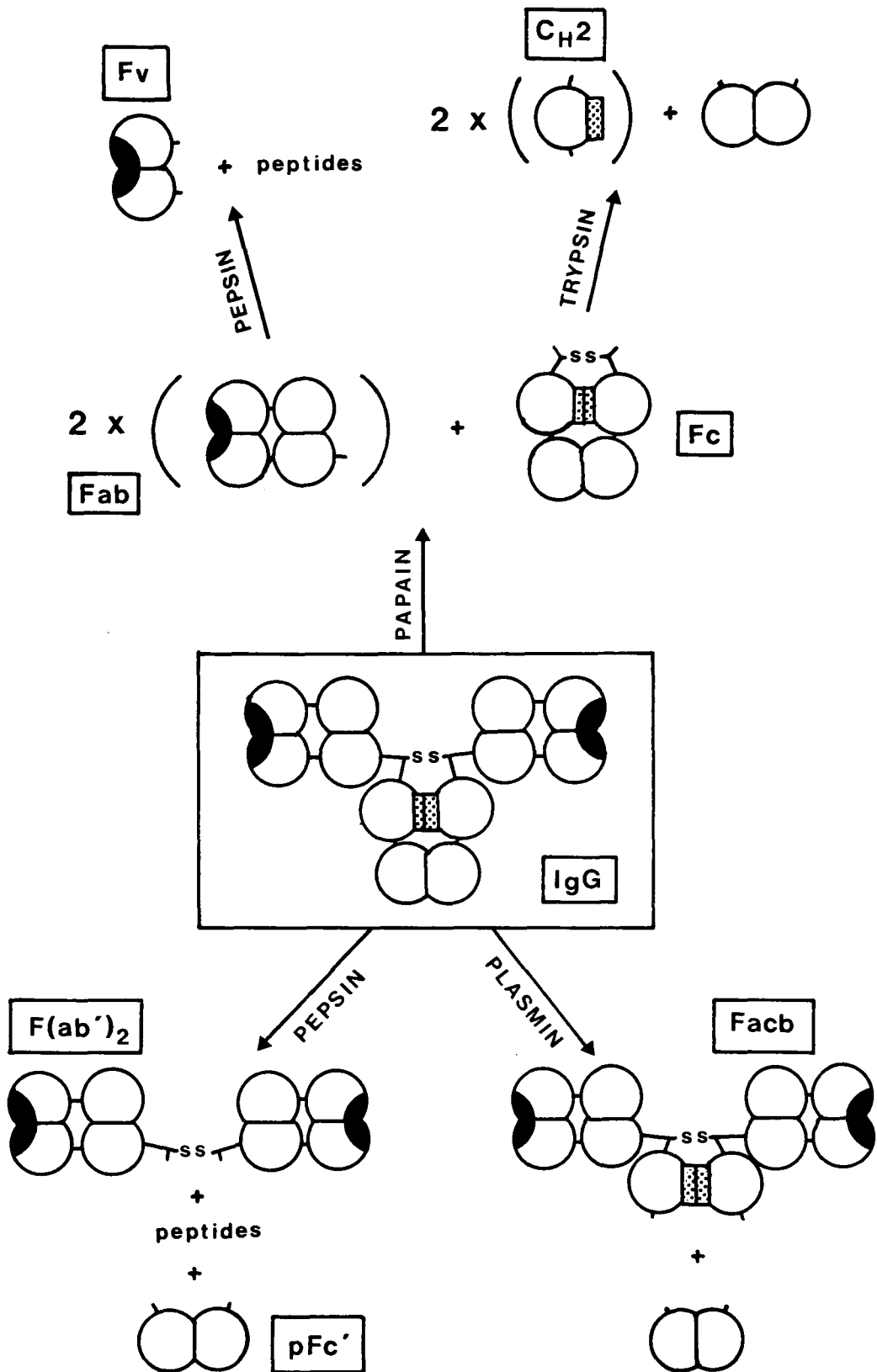


Figure 1.2 Schematic illustration of the major proteolytic fragmentation patterns of IgG (note that not all cleavages are possible with all IgGs).

cells, and is characterized by greatly elevated levels of homogeneous immunoglobulin in the circulation. Myelomas occur spontaneously in humans and most other mammals studied, and may be induced in mice by injection of mineral oils (Potter, 1972). Although several such proteins have been found to display antigenic activity comparable to naturally occurring antibodies (Eisen, 1968), the immunoglobulin produced usually has no known antigenic specificity.

2. Immunization with certain antigens occasionally produces a very restricted, essentially monoclonal response. Perhaps the best example of this is the response elicited in rabbit against various bacterial polysaccharide antigens (Osterland et al., 1966; Kimball et al., 1971). Since myelomatosis does not occur in rabbits this is the only method of producing a homogeneous antibody preparation with this species.

3. Fusion of myeloma cells with normal B cells can result in a stable hybrid cell (hybridoma) which produces the immunoglobulin of both parents and has the growth characteristics of the parent myeloma (Köhler & Milstein, 1975). Use of myeloma cell lines which have lost the capacity to produce immunoglobulin allows formation of hybridomas which produce only the immunoglobulin of the parent B cell (Köhler & Milstein, 1976; Köhler et al., 1976; Kearney et al., 1979). Therefore with a source of spleen cells from a suitably immunized mouse it is possible to select hybridomas producing antibodies of any required specificity. These may then be grown either in culture or in syngeneic mice to produce large quantities of homogeneous antibody. It is presently this technique which is the method of choice for producing immunoglobulins for structural studies.

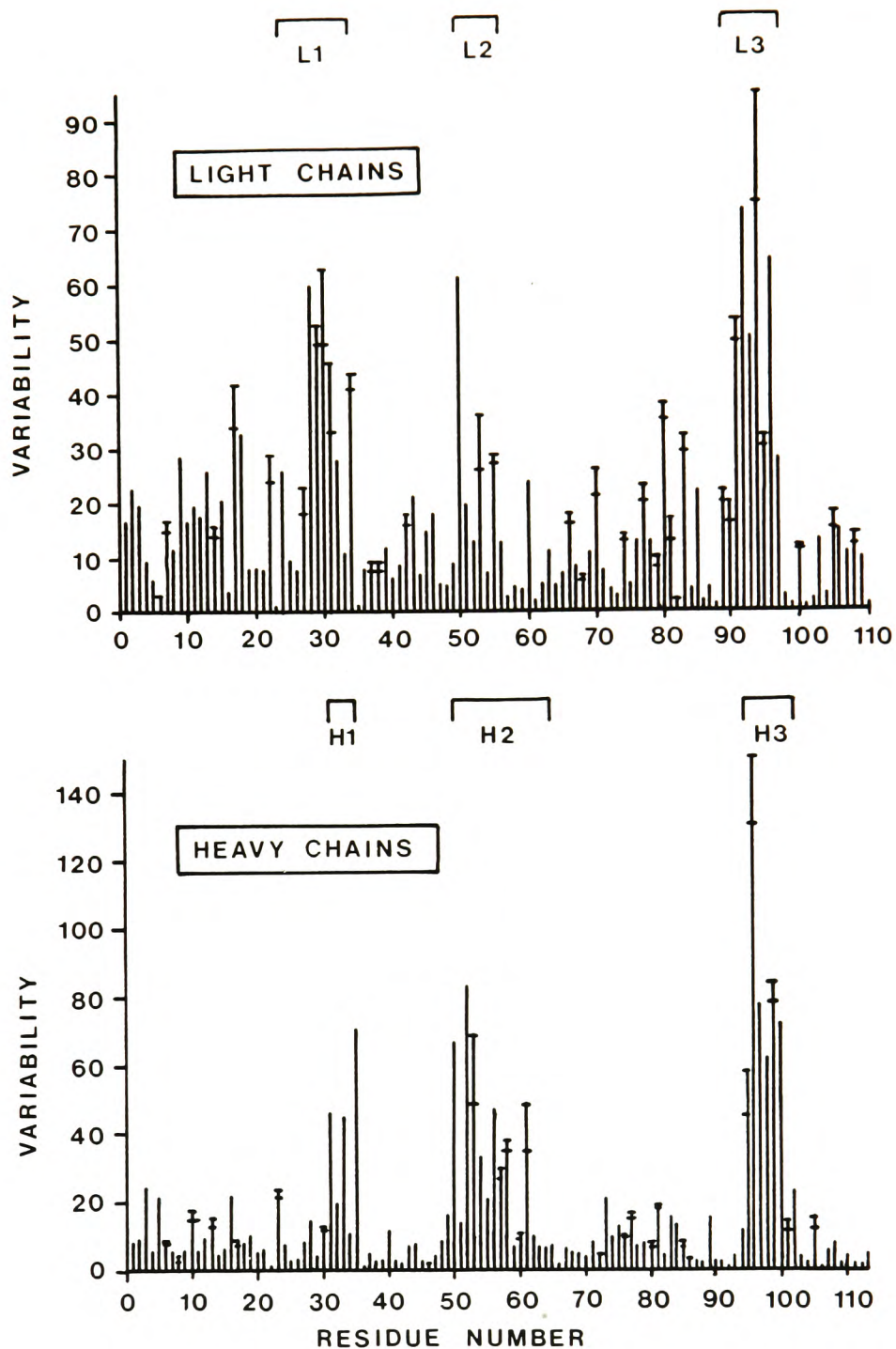


Figure 1.3 Variability of residues within the variable region of light and heavy chains of immunoglobulins (from Kabat *et al.*, 1979).

$$\text{Variability} = \frac{\text{number of different amino acids at a given position}}{\text{frequency of most common amino acid at this position}}$$

i.e. minimum = 1, maximum = 400. Horizontal bars indicate variabilities when values due to Glx or Asx are computed as only one of the two choices. The three hypervariable regions on each chain are indicated.

1.2.4 The antigen binding site

Within an isotype and allotype, the carboxy terminal domains (the constant or C domains) of an immunoglobulin have identical amino acid sequence. However the amino terminal domain (the variable or V domain) has in general a different sequence in every immunoglobulin chain examined. If the sequences of the V-regions are compared it is found that there are three distinct regions which show greater variability than the rest of the domain (Fig. 1.3). These hypervariable regions are located at residues 24-43, 50-56 and 89-97 of the light chain, and 31-35, 50-65 and 95-102 of the heavy chain. Proteolytic fragmentation (Inbar et al., 1972) and affinity labelling studies (reviewed by Givol, 1979) have shown that the antigen-binding region is located in the variable region, and is formed from these hypervariable portions of both the L and H chains.

Thus antibody diversity is a function of differing V-region sequences. This is possible due to the unique genetics of immunoglobulins (see Gearhart et al., 1981 and Givol et al., 1981 and the references cited therein). The whole immunoglobulin is coded for by three unlinked families of genes - the heavy chain, κ light chain, and λ light chain families. A whole chain is found to be coded for by the combination of one of many V-region genes present in the germ-line genome (although possibly further modified by somatic mechanisms) with one of the genetically linked C-region genes.

Table 1.1

Crystallographic structures of immunoglobulins and their fragments.

Protein	Species	Fragment	Resolution	Ligand	Ref
New	Human	Fab'	2.0 Å	vitamin K ₁ OH	1
McPC 603	Mouse	Fab'	2.7 Å	phosphocholine	2
Mcg	Human	L dimer	2.3 Å	DNP and others	3
REI	Human	V _L dimer	2.0 Å	DNP	4
Kol	Human	IgG	3.0 Å		5
Kol	Human	F(ab') ₂	1.9 Å		5
normal IgG	Human	Fc	2.9 Å		6
Dob	Human	IgG	6.0 Å		7
Rhe	Human	L dimer	3.0 Å		8
normal IgG1	Guinea Pig	pFc'	3.1 Å		9
normal IgG	Rabbit	Fc	2.7 Å		10

References:

1. Poljak et al., 1973, 1974; Amzel et al., 1974; Saul et al., 1978
2. Padlan et al., 1973; Segal et al., 1974; D. R. Davies, personal communication (see section 3.3.1)
3. Schiffer et al., 1973; Edmundson et al., 1974, 1975
4. Epp et al., 1974, 1975
5. Huber et al., 1976; Marquart et al., 1980
6. Huber et al., 1976; Deisenhofer et al., 1978; Deisenhofer, 1981
7. Silverton et al., 1977
8. Wang et al., 1979
9. Phizackerley et al., 1979
10. Sutton & Phillips, 1983, and personal communication

1.3 THREE-DIMENSIONAL STRUCTURE OF IMMUNOGLOBULINS

Many excellent reviews of immunoglobulin three-dimensional structure are available and so the following section only briefly covers the most important aspects which are of relevance to subsequent chapters. A fuller treatment is found in Davies et al. (1975a,b), Poljak (1975, 1978), Padlan & Davies (1975), Beale & Feinstein (1976), Givol (1979), Lesk & Chothia (1982), and the references listed in Table 1.1, particularly Edmundson et al. (1975). The nomenclature used for the various structural features of the immunoglobulin domains is that of Beale & Feinstein (1976).

1.3.1 Domain structure

Numerous crystallographic studies on immunoglobulin fragments (summarized in Table 1.1) have shown that each of the repeating homology regions exists as a distinctly folded unit, giving striking confirmation of the domain hypothesis. Each domain consists of two stacked antiparallel β -sheets, one comprising three and the other four amino acid strands (Fig. 1.4). These seven roughly linear segments are joined by loops of various structure and length. Each of the β -sheet 'faces' are extensively hydrogen bonded, and the three-strand face (face Y) is linked to the four-strand face (face X) by an intra-chain disulphide bridge. The inter-facial region is filled by hydrophobic amino acid side chains. This characteristic folding pattern is termed the immunoglobulin fold.

Three residues are common to all immunoglobulin domains: the two cysteine residues forming the intra-chain disulphide; and a tryptophan

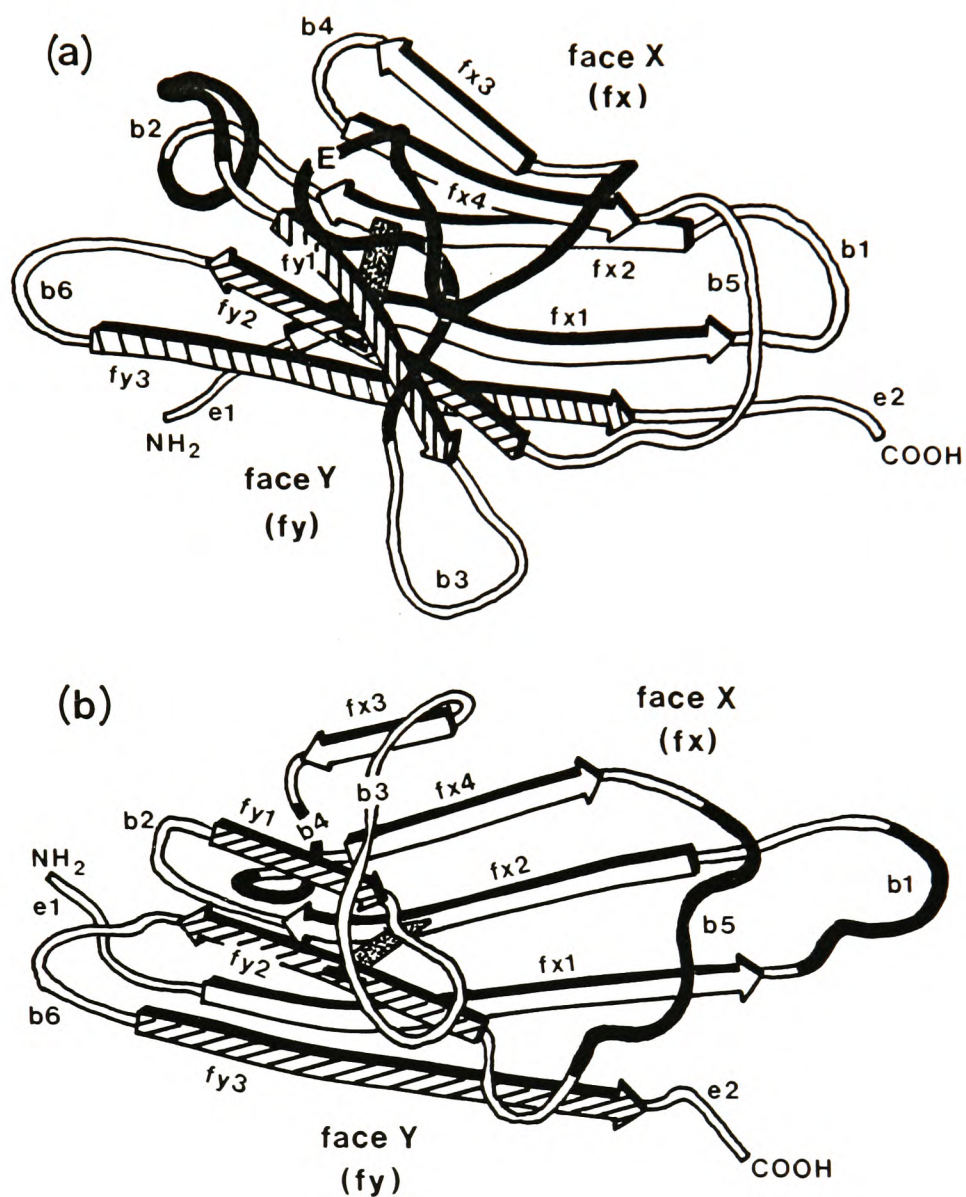


Figure 1.4 Immunoglobulin domain structure

(a) variable region domain

(b) constant region domain

From Beale & Feinstein (1976). Positions where differences occur between the constant and variable domains are indicated by solid black lines. The intra-chain disulphide bridge between fx2 and fy2 is shown.

residue which is also part of the β -sheet structure, and which is found to pack its side chain against this disulphide. These residues form the basis of a central highly-conserved structural sub-region of the domain, which Lesk & Chothia (1982) designate 'the pin'.

Although these basic features are present in the immunoglobulin domains of all classes so far examined, it is found that while the constant region domains display remarkable similarities to each other, the V-regions differ slightly. Amongst other differences (discussed in much greater detail by Edmundson *et al.* (1975) and shown by solid black lines in Fig. 1.4) the V-region β -sheet faces are more distorted than those of the C-domains, and they possess an additional loop between the fyl and fx3 strands (see Fig. 1.4). This additional loop effectively gives an additional two strands to the three strand Y face. The hypervariable regions (section 1.2.4) constitute loops b6, b4, and the analogous bend E of Fig. 1.4a and are not integral to the structural β -strand segments of the immunoglobulin fold. When different V-domain structures are compared it is found that while the β -sheet folding structure is conserved, different hypervariable loops, while attached at conserved positions, are folded differently (Padlan & Davies, 1975). As is discussed further in section 1.10.3 and 3.1.1, this leads to the concept of the variable region as a framework onto which the different hypervariable regions are attached. This is the molecular basis for the generation of diversity in the immune response.

1.3.2 Quaternary three-dimensional structure

As is indicated from the chain structure of immunoglobulins a domain on one chain exists paired with one on the neighbouring chain.

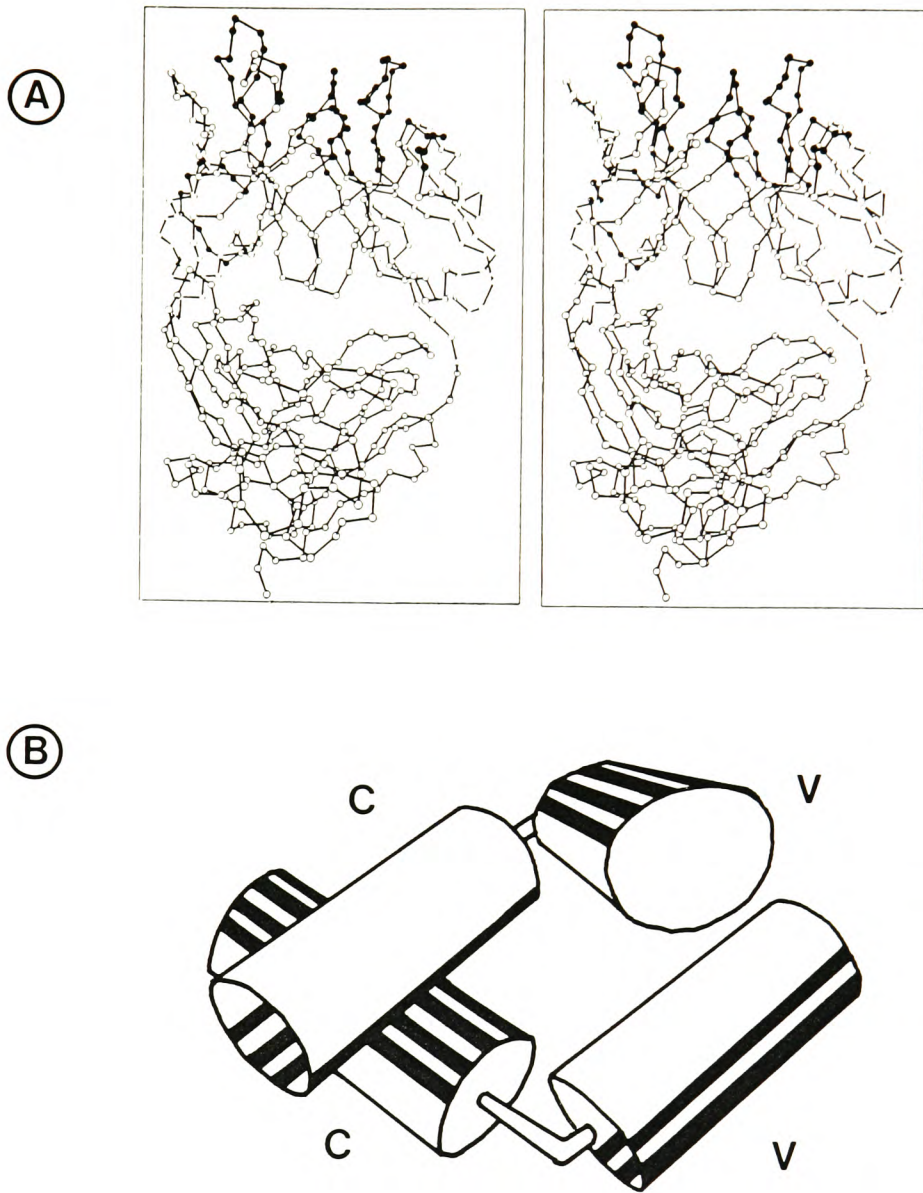


Figure 1.5 Structure of an Fab fragment

- (a) Stereo view of the Fab' fragment of McPC 603 (from Davies et al., 1975b). The hypervariable loops are indicated by filled circles.
- (b) Schematic illustration of the different pairing of the V and C domains of Fab. The four-strand X faces are shaded (from Edmundson et al., 1975).

Of the four domain-pair types which constitute IgG, three different pairings are found.

1. X-X face pairing. This is found in both the $C_{H1}-C_{L1}$ and $C_{H3}-C_{L3}$ pairs (Fig. 1.5 and 1.6). Extensive non-covalent cis interactions exist between the X faces of these domains. In addition the C_{H1} and C_{L1} pair is (usually) linked by a disulphide bond (Fig. 1.1).

2. Y-Y face pairing. The V-regions are found to interact non-covalently via their Y faces. This is possibly favoured by the additional loop found in the Y face of these domains (section 1.3.1) which participates in such pairing. Fig. 1.5 shows the Fab' fragment of McPC 603 IgA. The V-region pairing is such that the three hypervariable loops of each domain (shown by filled circles in Fig. 1.5a) are brought together to form the binding site cleft. The C_{H1} and C_{L1} domains associate via their X faces.

There is found to be very little longitudinal interaction between the V and C domains of Fab, which are joined by a linking polypeptide strand termed the "switch" region. Some potential for flexibility is associated with this region resulting in slightly different "elbow-bend" angles for the various Fab and L_2 fragments studied.

3. C_{H2} domain-like pairing. When the structures of the Fc fragments of human (Deisenhofer, 1981) or rabbit (B. Sutton & D. C. Phillips, personal communication) IgG are examined, a striking feature is that there are no protein-protein cis interactions between the two C_{H2} domains (Fig. 1.6). Instead they are separated, and the interstitial region is filled with a branched oligosaccharide attached to Asn-297 and

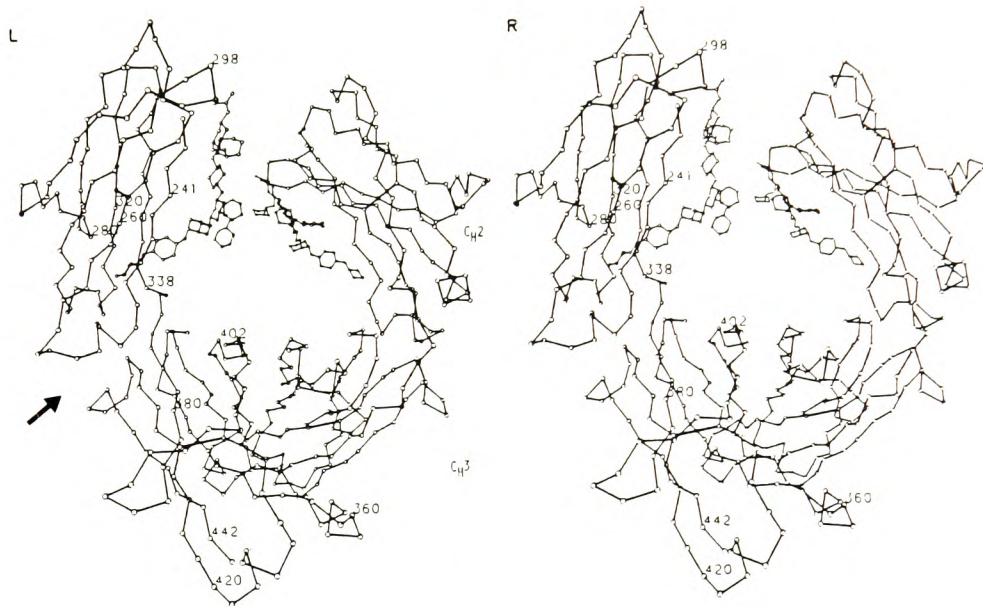


Figure 1.6 Stereo view of the Fc fragment of human IgG (from Deisenhofer, 1981). Shown are the alpha-carbon atoms of the protein, plus the carbohydrate (hexose rings, glycosidic linkages, and Asn-297 side chain). The protein A binding region is arrowed.

covering the X face of each domain. Therefore the only interactions between these domains are via carbohydrate-carbohydrate contacts. The C_H^3 domains however, have the same X-X face pairing as the constant regions of Fab. That the C_H^2 - C_H^2 interaction is weak is evidenced by the isolated C_H^2 fragment of IgG existing as a monomer, in contrast to the dimeric $(C_H^3)_2$ fragment (Ellerson et al., 1976), and by the reduced and alkylated Fab_b fragment dissociating into two halves while similarly treated IgG does not (Johanson et al., 1981). Whether the two C_H^2 domains remain closely associated when the hinge disulphide is reduced in whole IgG is controversial. Segan et al. (1979) report no difference in shape on reduction and alkylation, Pilz et al. (1980) report a slight effect on overall shape, and Luedtke et al. (1981) calculate a separation of the two halves of the hinge of 50-60 Å. The associations of C_H^1 with C_L and V_H with V_L appear to be intermediate in strength to those of the C_H^2 and C_H^3 pairs, however the combination of the two relatively weak interactions of the former two pairs results in a strong overall Fd-L association (Klein et al., 1979).

Slightly more extensive longitudinal interactions are found between the C_H^2 and C_H^3 domains than between the V and C domains of Fab, and there is no real equivalent separating switch peptide. Some potential for flexibility must exist at this point however, as it has been shown that when the hinge disulphide is reduced a pseudo-hinge may be formed between the C_H^2 and C_H^3 regions when IgG interacts with a multivalent antigen (Romans et al., 1977).

The only whole immunoglobulin whose full crystal structure has been determined (Kol) displays a unique feature in that no interpretable electron density is found for the Fc region (Huber et al., 1976;

Marquart et al., 1980). This phenomenon has also been found for another intact IgG (Zie) although no high resolution data are available (Ely et al., 1978). This effect is explained by the hinge region conferring flexibility to the molecule. A hinge-deleted immunoglobulin has been studied (protein Dob) (Silverton et al., 1977; Steiner & Lopes, 1979) and shows Fab and Fc regions with the same structure as the individual fragments, and closely associated to form an overall "T" shape. Presumably the deletion makes the molecule sufficiently rigid to allow the Fc region to adopt a more defined conformation. The flexibility at the hinge region is clearly visible in electron micrographs (Valentine & Green, 1967), which suggest an average "Y" shape for the molecule, a shape also inferred from the Fab-Fab angle in the crystal structure of Kol (Huber et al., 1976; Marquart et al., 1980). Solution small-angle X-ray scattering studies, however, find that the scattering curves are more consistent with a "T" (Kratky & Pilz, 1978). The lack of crystallographic data for an intact IgG makes it difficult to comment on the interaction between the Fab and Fc regions, or how differing hinge length (very pronounced between subclasses in human IgG) alters their relative disposition. Although it is often assumed that the hinge region is extended (Marquart et al., 1980), one interesting recent study using small angle X-ray scattering suggests that this may not be the case. It is found that IgG3 has a smaller radius of gyration than IgG1, indicating that its hinge does not exist as a long spacer but compacted between the Fab and Fc regions (I. Simon, F. Kilár & P. Závodszky, personal communication).

1.4 IMMUNOGLOBULIN CARBOHYDRATES

1.4.1 Position of glycosylated sequences

All immunoglobulins are glycoproteins, i.e. they contain covalently attached carbohydrate residues. The extent of glycosylation is essentially class specific and ranges from 2-3% in IgG to ~ 12% in IgE and IgM, representing between 1 and ~ 6 N-linked units per heavy chain of the molecule. In general the sugar residues are all attached to the heavy chain constant regions, however specific myeloma proteins (and possibly a small proportion of naturally occurring antibodies) have variable region glycosylation. These will be dealt with separately. The light chain constant region is not glycosylated.

Constant region glycosylation. Although all classes of immunoglobulins are N-glycosylated, O-glycosylation has only been reported in one subclass of human IgA, in an allotype of rabbit IgG, and in human and mouse (Vasilov & Ploegh, 1982) IgD (see Fig. 1.7). In all these instances it is the hinge region which contains the O-linked sugar(s). The distribution of N-linked oligosaccharides is more varied, as is illustrated in Fig. 1.7. It is found that within a class of immunoglobulin there is often a characteristic glycosylation pattern. Between classes there are also highly conserved glycosylation positions. In particular the glycosylation site at Asn-297 (Eu numbering, equivalent to Asn-402 in Ou numbering) has been found in all γ , δ , ϵ and μ chains of all species so far examined. Secreted immunoglobulins containing a tail piece which is involved in multimeric assembly (α and μ chains) are always glycosylated at the equivalent residue to Asn-563 (Ou numbering). Conservation of an amino acid

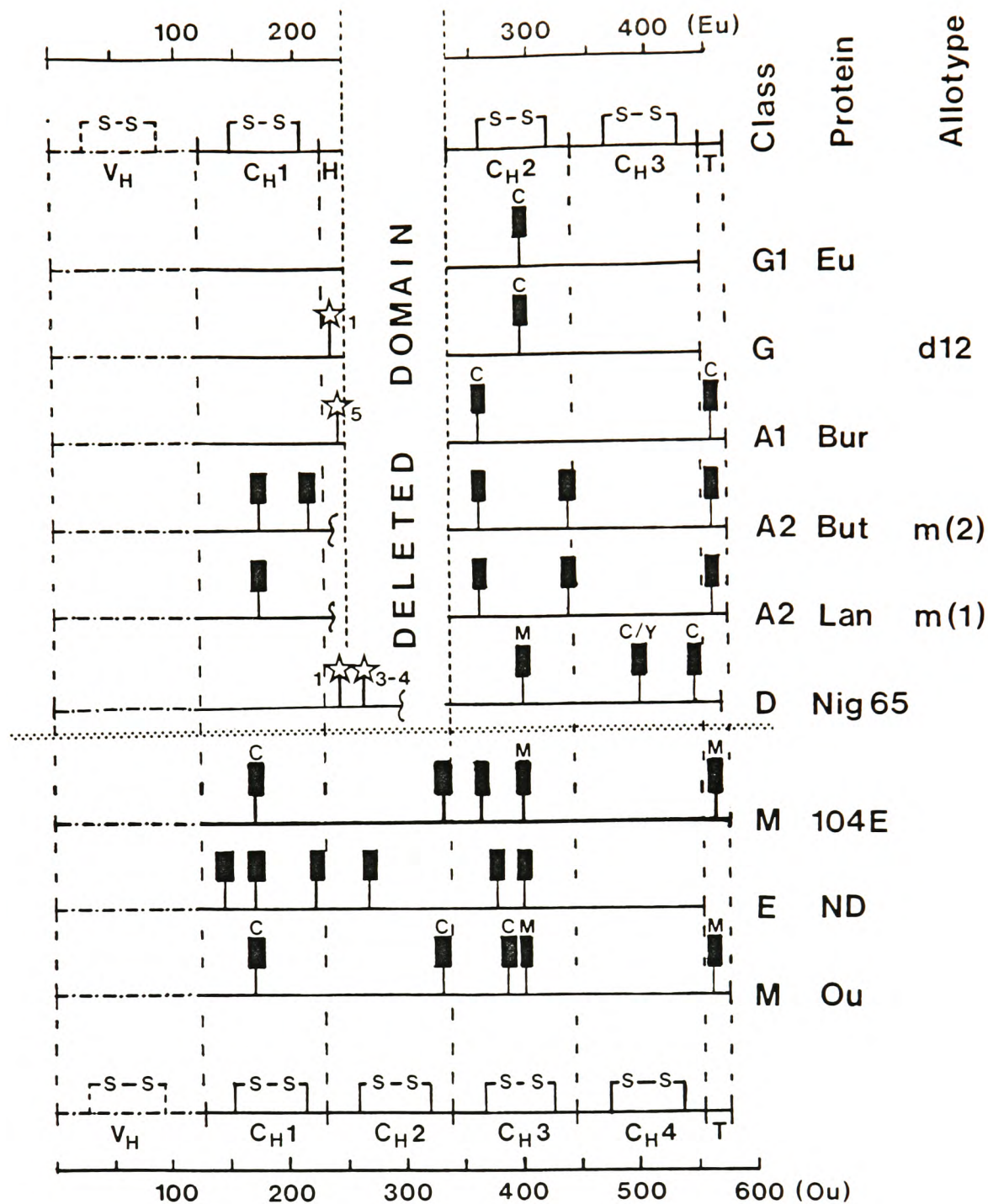


Figure 1.7 Location of constant-region glycosylation positions

■ Asparagine-linked oligosaccharide. Type (C = complex, M = high mannose, Y = hybrid) is indicated if known.

★ O-linked oligosaccharide. If several occur very close together the number is placed beside the symbol.

Compiled from: Smyth & Utsumi, 1967; Fanger & Smyth, 1972; Shimizu et al., 1971; Putnam et al., 1973; Baenziger & Kornfeld, 1974; Beale & Feinstein, 1976; Torano et al., 1977; Kabat et al., 1979; Kehry et al., 1979; Tsay et al., 1980; Lin & Putnam, 1981; Putnam et al., 1981; Shinoda et al., 1981; Ishihara et al., 1983.

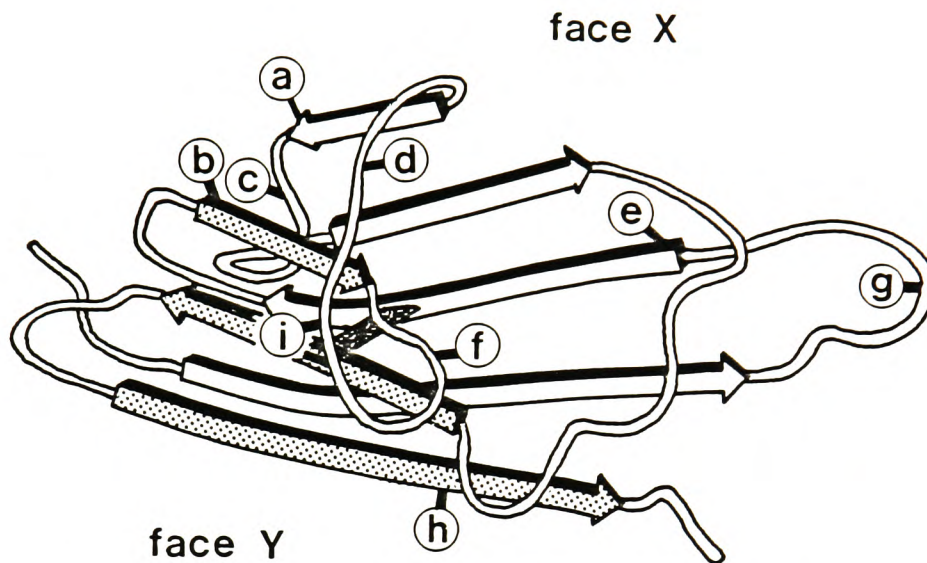


Figure 1.8 Location of oligosaccharide sites on the three-dimensional structure of a C domain (adapted from Beale & Feinstein, 1976 with additional data from Toraño *et al.*, 1977; Kehry *et al.*, 1979; Lin & Putnam, 1981). Glycosylation sites in the hinge region and tail piece are omitted (see Fig. 1.7).

IgG has site (c) in the $C_{\gamma}2$ domain.

IgM (human) has site (f) in the $C_{\mu}1$ domain; (h) in $C_{\mu}2$; (a) and (c) in $C_{\mu}3$. Mouse IgM lacks site (a) in $C_{\mu}2$, but has site (e) in $C_{\mu}2$.

IgA1 has site (e) in $C_{\alpha}2$. IgA2 has additionally site (h) in $C_{\alpha}2$; and (d) in $C_{\alpha}1$. IgA2 (allotype m2) has also (h) in $C_{\alpha}1$.

IgE has sites (g), (d) and (h) in $C_{\epsilon}1$; (b) in $C_{\epsilon}2$; and (c) in $C_{\epsilon}3$.

IgD has site (c) in $C_{\delta}2$; (i) and (h) in $C_{\delta}3$.

sequence (in this case the triplet sequence -Asn-Xaa-Ser/Thr- required for N-glycosylation [Marshall, 1974]) indicates that it may impart some functional advantage. This is particularly the case if the sequence is conserved between classes. The possible functions of this glycosylation form part of this thesis and are discussed further in the relevant chapter (chapter 6).

Variable region glycosylation. Numerous reports have indicated that myeloma proteins occasionally contain additional carbohydrate located in the V domains. Abel et al. (1968) reported that 29% of the IgG myeloma proteins which they examined (76 in total) contained carbohydrate in the Fab region. In a study of light chain glycosylation 2 out of 18 myeloma proteins and 3 out of 71 Bence-Jones proteins (i.e. ~ 6% of the total light chains studied) were N-glycosylated (Sox & Hood, 1970). The oligosaccharide is found to be located in the hypervariable regions. Carbohydrate has been reported to occur on all three hypervariable regions of the light chain, and the first two such regions of the heavy chain (Press & Hogg, 1970; Sox & Hood, 1970; Spiegelburg et al., 1970; Chandrasekaran et al., 1981; Savvidou et al., 1981). Presumably this represents the chance occurrence of an -Asn-Xaa-Ser/Thr- glycosylation acceptor sequence (Marshall, 1974) when forming the hypervariable regions. The structures of these V-region N-linked oligosaccharides seem to be of the same complex-type as in the C_γ2 domain (Chandrasekaran et al., 1981; Mizuochi et al., 1982) (see section 1.4.3). Although V-region carbohydrate is usually N-linked, there is a report of an O-linked sugar occurring in the first hypervariable region of an abnormal light chain (Chandrasekaran et al., 1981). To the authors knowledge the only antibody reported to have both antigenic activity and V-region glycosylation is MOPC 104E which has a sixth glycosylation site at

Asn-57_H (Kehry et al., 1979). This conflicts however with the data of Brenckle & Kornfeld (1980) who find only five N-glycosylation positions, and also occurs at a reported -Asn-Gly-Gly- sequence which is incompatible with the requirements of the glycosylation pathway (Marshall, 1974; Struck & Lennarz, 1980). The report in Kehry et al. (1979, "unpublished observations") of other anti-(1-3)-dextran antibodies having a similar glycosylation site has not been confirmed.

1.4.2 Location of the constant domain glycosylation points

The locations of the N-glycosylation positions on the three-dimensional model of an immunoglobulin C-domain are shown in Fig. 1.8. With the exception of site (e), all the glycosylation positions are on or very close to the bend regions which join the antiparallel β -sheets. Assuming an X-X face pairing interaction between domains (see section 1.3.2) it is found that with three exceptions all the carbohydrate attachment sites are such that the oligosaccharide will lie on the exterior face (Y) of the domain pair. These exceptions are sites (a), (c) and (e) of Fig. 1.8 which occur on the X-face of the C $_{\alpha}$ 2, C $_{\gamma}$ 2 and C $_{\delta}$ 2, and homologous C $_{\epsilon}$ 3 and C $_{\mu}$ 3 domains. In IgG this domain-pair shows no X-X face interactions, instead the X-face oligosaccharide occupies the region between the two C $_{\gamma}$ 2 domains (see Fig. 1.6). It is reasonable to suppose that the homologous glycosylation site (Asn-297, Eu numbering) found in all other classes except IgA indicates that a similar type of protein / oligosaccharide "sandwich" structure exists in these classes, and probably accounts for the extreme conservation of this glycosylation site. Although this site does not exist in IgA, the X-face is still N-glycosylated (at Asn-258) and it is therefore probable that X-X face interactions do not occur in the penultimate domain of any

of the immunoglobulin classes.

Fig. 1.8 also shows that certain domain positions are frequently glycosylated even though the glycosylation does not necessarily occur on strictly homologous domains. Thus position (h) is glycosylated in $C_{\mu}2$, $C_{\alpha}2$ (IgA2), $C_{\alpha}1$ (IgA2 allotype m2), $C_{\epsilon}1$, and $C_{\mu}3$. The significance (if any) of this is not known.

1.4.3 Structure of the N-linked oligosaccharides of IgG

It is only comparatively recently that it has become possible to rapidly and accurately determine the complete sequence of the large complex-type oligosaccharides found on glycoproteins. Therefore while much early work was performed on the structure of immunoglobulin carbohydrates the exact sequences reported may not be correct, although they will probably give an indication of the likely structure. Thus Kornfeld et al. (1971) report sequences for human IgG oligosaccharides which although approximating to those described in the more recent work of Mizuochi et al. (1982) contain structures which differ from both this later work and the proposed biosynthetic pathway for asparagine-linked sugar chains (Struck & Lennarz, 1980). In the light of this the most reliable structures for IgG are likely to be those recently determined, i.e. human IgG (Mizuochi et al., 1982) and rabbit IgG (Rademacher et al., 1983, and personal communication). In both of these proteins quite a large mixture of oligosaccharides of different length are found. All of these are produced by differing degrees of incompleteness of a single sugar chain, although the relative proportions of each form vary between these species (and between different human myeloma IgG samples). The data for human IgG is depicted in Fig. 1.9. This

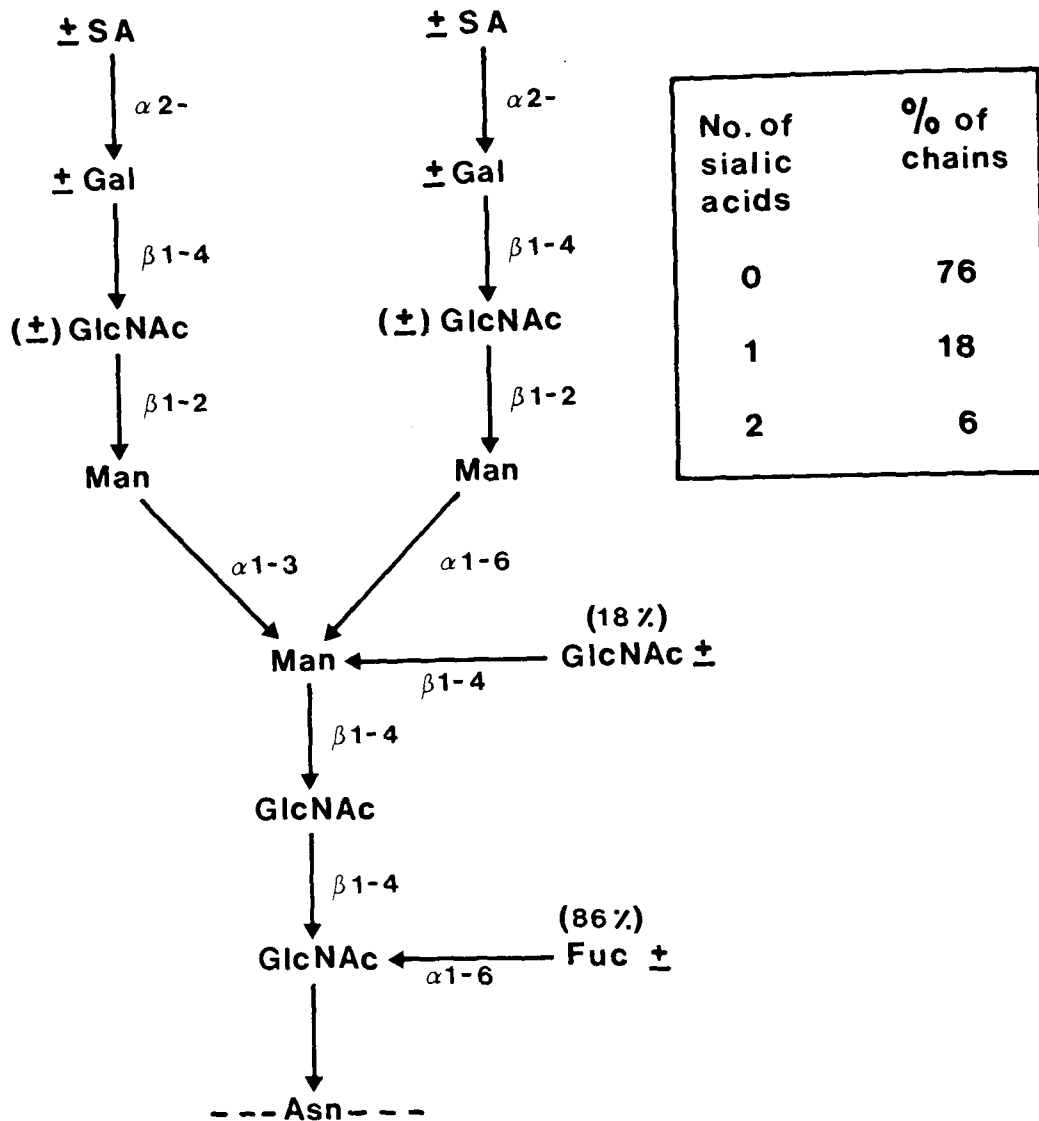


Figure 1.9 The structure of the N-linked oligosaccharides of human IgG (Mizuochi et al., 1982).

± Missing in a proportion of the chains. The percentage of chains with this residue present is given in parentheses if known.

(±) Missing in a proportion of the chains in some of the myeloma IgGs studied.

structure is also found in human IgA1 (Baenziger & Kornfeld, 1974) and on mouse IgG (Rademacher et al., personal communication), including more specifically the IgG2a produced by the K3 cell line which is used extensively in this thesis.

The relatively high proportion of terminal galactose residues in IgG is interesting since exposure of such residues leads to rapid clearance via the hepatic galactose receptor (see sections 1.9.1 and 6.1.1). Since the serum half-life of IgG is long (Table 1.2) it indicates that the galactoses are not highly exposed. This is consistent with the semi-buried location of the oligosaccharide found in the crystal structure of Fc (Fig. 1.6).

Table 1.2 Some of the functional properties of immunoglobulins.

	normal conc (mg/ml)	half- life (days)	Complement		PCA	Cell binding			SpA
			Clas.	Alt.		MC	P'let	NP	
<u>Human</u>									
IgG1	9	21	+	-	+	+	+	+	+
IgG2	3	20	<u>+</u>	-	-	<u>+</u>	+	+	+
IgG3	1	7	+	-	+	+	+	+	-
IgG4	0.5	21	-	-	+	<u>+</u>	+	+	+
IgA1	3	6	-	+	-	-	-	+	-
IgA2	0.5	6	-	+	-	-	-	+	-
IgM	1.5	5	+	-	-	-	-	-	-
IgD	0.03	3	-	<u>+</u>	-	-	-	-	-
IgE	0.00005	2	-	<u>+</u>	-	-	-	-	-
<u>Mouse</u>									
IgG1	3.7	4	-	-	+	+			<u>+</u>
IgG2a	0.8	5	+	-	-	+			+
IgG2b	0.5	2	+	-	-	+			+
IgG3	0.1	4	-	-	-	+			+
IgA	0.3	1	-	+	-	-			-
IgM	0.04	1	+		-	-			-
IgE			-		+				-

Clas. = Classical Alt. = Alternative NP = Neutrophil

PCA = Passive cutaneous anaphylaxis P'let = Platelet

MC = Monocyte SpA = Staphylococcal protein A binding

From Grey et al., 1970; Spiegelberg, 1974; Ey et al., 1978; Stanworth & Turner, 1978.

1.5 FUNCTIONAL CHARACTERISTICS OF IMMUNOGLOBULINS

Immunoglobulins are multi-functional proteins - as well as binding antigens they interact with one or more secondary systems. The characteristic interactions of the various classes / subclasses of human and murine immunoglobulins are shown in Table 1.2. Since part of this thesis concerns the possible role of the oligosaccharide of mouse IgG in these secondary functions, the following sections (1.6 to 1.9) describe the features of the interactions which are relevant to IgG.

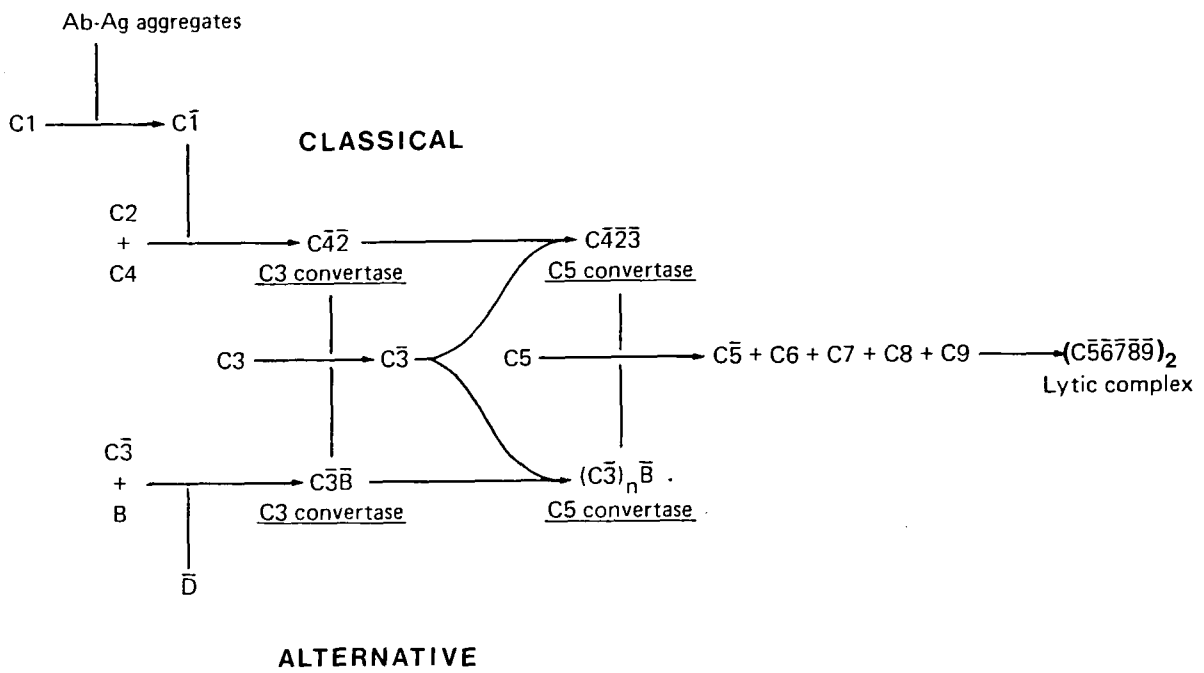


Figure 1.10 Schematic diagram of the classical and alternative pathways of the complement cascade (from Porter, 1979; Biesecker et al., 1979).

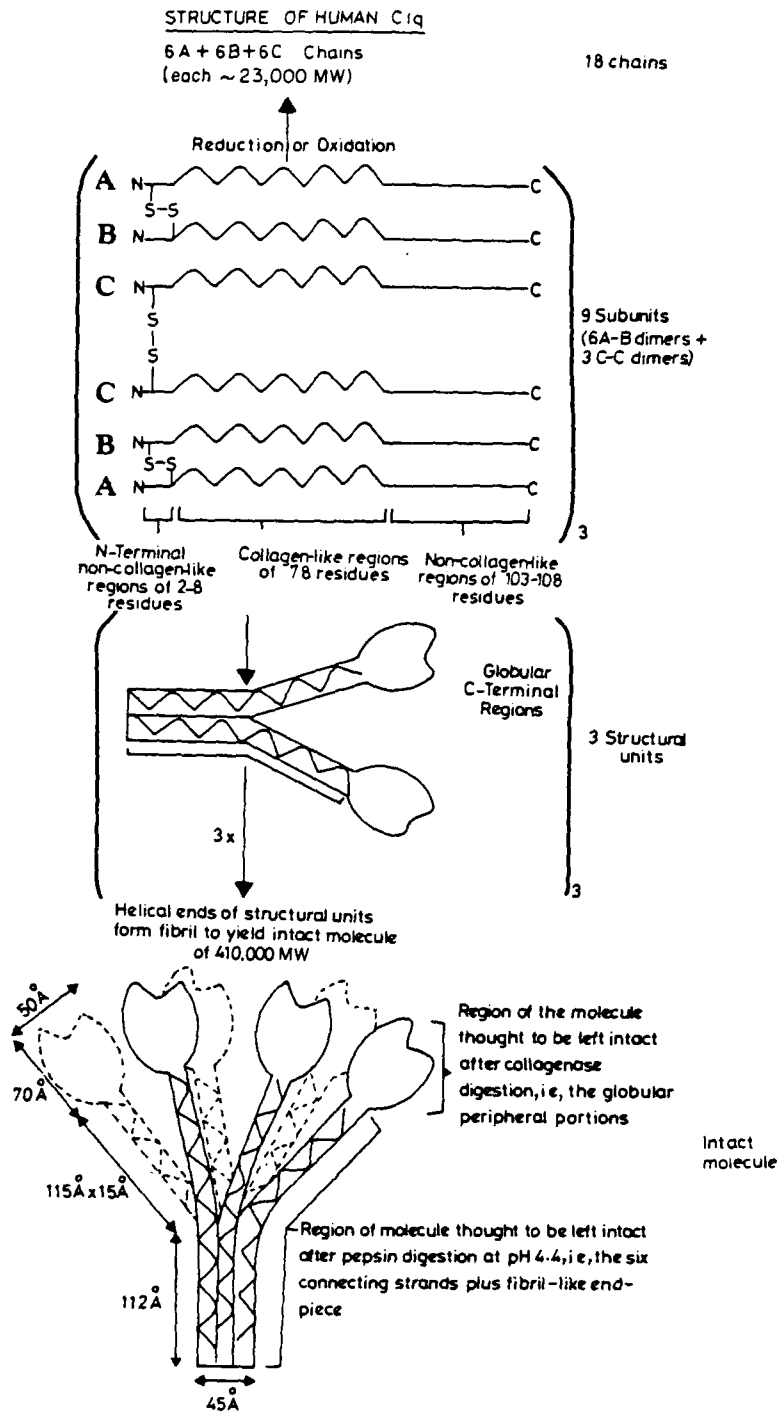


Figure 1.11 The structure of human C1q (from Porter & Reid, 1979). Dimensions are averages of those published by Shelton *et al.* (1972) from electron microscopy. Wavy lines represent proposed triple helix sections, i.e. collagen-like regions of the molecule. Length of collagen-like fiber + fibril-like end piece = $11.5 + 11.2 = 22.7$ nm. Length of triple helix from sequence studies = $80 \times 0.29 = 23.2$ nm.

N-linked oligosaccharide occur in the globular heads (Mizuochi et al., 1978); and ~ 67 mol/mol of Glc α 1-2Gal β hydroxylysine are found in the tails (Shinkai & Yonemasu, 1979; Bornstein & Traub, 1979) i.e. ~ 80% of the hydroxylysines are glycosylated. This glucosylgalactosylation, together with the unusual amino acid composition, makes this protein reminiscent of collagen. Electron microscopic studies reveal an equally unique shape: six peripheral globular regions are found, each attached via a connecting strand to the end of a thicker central strand (Shelton et al., 1972; Knobel et al., 1975). Amino acid sequence studies predict that the strand-like regions resemble collagen in that they are formed from the Gly-X-Y repeated triplet sequence found in the N-terminal region of all three chains of Clq. Discontinuities in this triplet sequence in the A and C chains, and an extra triplet in the B chain, are expected to cause a distortion in the triple helix at the point corresponding to the connecting strand-central strand join seen in the electron micrographs (Porter & Reid, 1979). The resultant structure, assuming a stoichiometry (ABC)₆ is shown diagrammatically in Fig. 1.11. This model is also supported by studies involving use of circular dichroism and enzymic digestion (Brodsky-Doyle et al., 1976).

The electron micrographs of Clq indicate that the molecule is very flexible, with the angle of the Clq arms from the mid-line being on average ~ 50° (Schumaker et al., 1981). Solution studies (Liberti & Paul, 1978; Gilmour et al., 1980) are consistent with this arrangement and confirm that the Clq stalks adopt on average an open conformation. However, a preliminary report of nanosecond polarization of fluorescence measurements on Clq indicates that in solution the flexibility of the molecule may not be as great as suggested by the electron micrographs (Hanson et al., 1981).

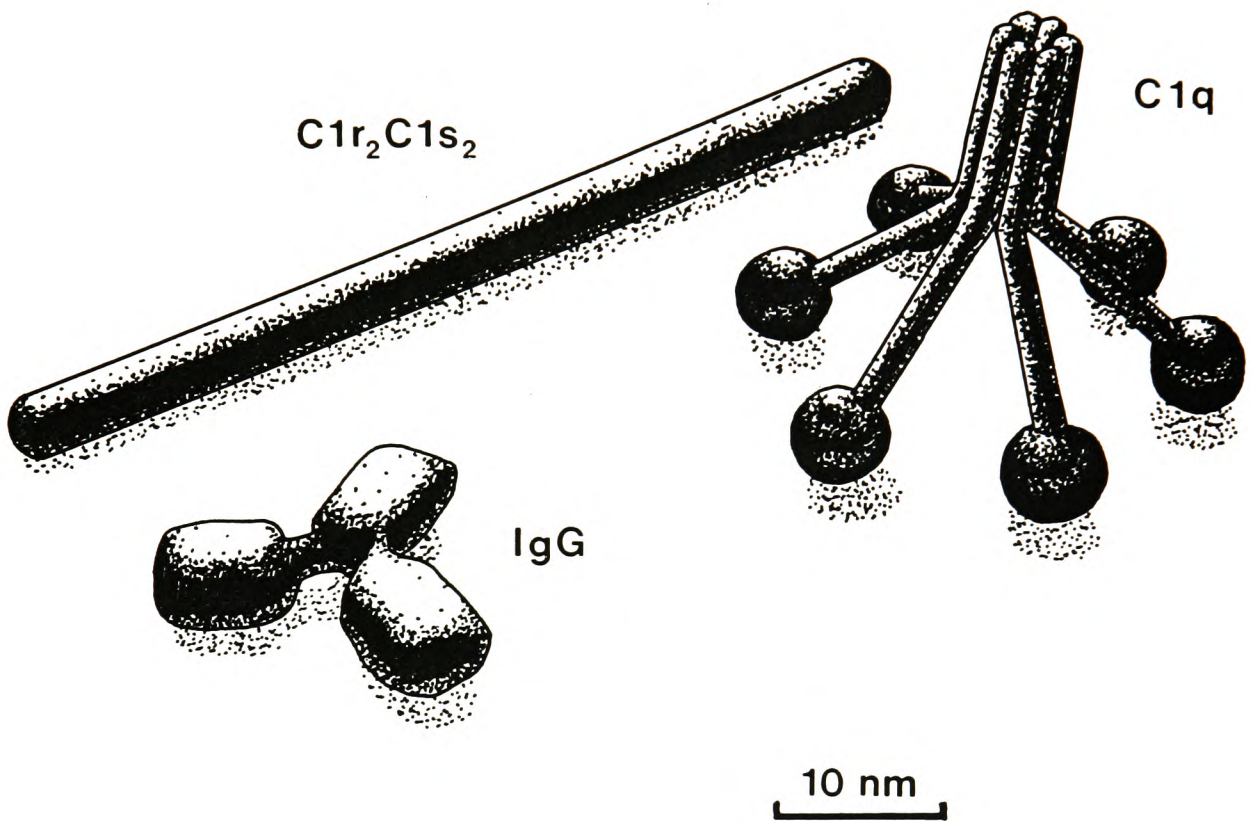


Figure 1.12 Scale diagram illustrating the relative size and shape of C1q, C1r₂C1s₂ and IgG as found from electron microscopy, X-ray crystallography and solution neutron scattering studies.

Subcomponents Clr and Cls: The general features of these two sub-components are very similar (reviewed by Porter & Reid, 1979; Sim, 1981). Both occur in serum as proteolytic zymogens and comprise a single ~ 83,000 dalton polypeptide chain. In the presence of Ca^{2+} they form a tetrameric complex of Clr_2Cls_2 . Electron microscopy, hydrodynamic measurements and small-angle neutron scattering studies indicate that this complex has an extended rod-like structure with dimensions of 59 x 3.8 nm (Tschopp et al., 1980b; Strang et al., 1982; Boyd et al., 1983). Activation involves a single cleavage of the peptide chain without apparent loss of molecular weight to produce typical serine proteases (Sim et al., 1977). $\bar{\text{Cl}}\text{s}$ is thought to be responsible for activation of C4 and C2, the next components in the pathway, as $\bar{\text{Cl}}\text{r}$ does not act on them in solution, although it will hydrolyse Cls (Porter & Reid, 1979). The shape, and relative sizes of Clq and Clr_2Cls_2 in relation to IgG are depicted in Fig. 1.12.

Structure of component Cl: Although electron microscopy studies on Clq have given much information on its structure, similar studies on Cl have been less successful - apparently due to dissociation of the complex during sample preparation (Tschopp et al., 1980b). Chemically cross-linked Cl has been used to circumvent this problem and has provided reasonable micrographs (Strang et al., 1982), however it is not known to what extent this cross-linking introduces artifacts. The model for Cl which emerged from this work is that Clr_2Cls_2 is positioned on the thin connecting fibrils of Clq between the heads and the central bundle. It is not possible to discern whether the Clr_2Cls_2 occurs on the exterior or interior of these fibrils, or indeed interwoven in between them as favoured by the authors. It was also observed that this Cl exhibited reduced flexibility compared with Clq (V. N. Schumaker,

personal communication), although the contribution of the cross-linking agent to this is not possible to ascertain.

1.6.3 The binding of C1 to antibody/antigen complexes

Of the five classes of immunoglobulins, only IgM and certain subclasses of IgG activate the classical pathway of complement. In humans the IgG subclasses which fix complement are IgG1 and IgG3; IgG2 fixes more weakly, and IgG4 does not fix at all (Augener et al., 1971). This correlates with the ability of these subclasses to bind to Clq (Augener et al., 1971; Schumaker et al., 1976). It is well-established that the interaction is between the globular heads of the Clq subunit of C1 (Knobel et al., 1974; Hughes-Jones & Gardner, 1979) and the Fc region of the immunoglobulin (Taranta & Franklin, 1961; Augener et al., 1971; Sledge & Bing, 1973). The exact location of the site on Fc is somewhat controversial (see section 1.6.4) although its localization to the C_γ2 domain of IgG is generally accepted (Kehoe & Fougereau, 1969; Colomb & Porter, 1975; Ovary et al., 1976; Yasmeen et al., 1976). In IgM the Clq site may reside in the C_μ4 domain rather than the homologous C_μ3 domain (Hurst et al., 1975; Bubb & Conradie, 1978).

1.6.4 Location of the Clq binding site in the C_γ2 domain

The exact location of the Clq binding site in the C_γ2 domain of IgG is still unclear, despite a wide variety of approaches to attempt a structural definition of the site, and detailed knowledge of the structure of both human (Deisenhofer et al., 1978; Deisenhofer, 1981) and rabbit (B. J. Sutton & D. C. Phillips, personal communication) Fc fragments. It is generally accepted that the site is quite ionic, on

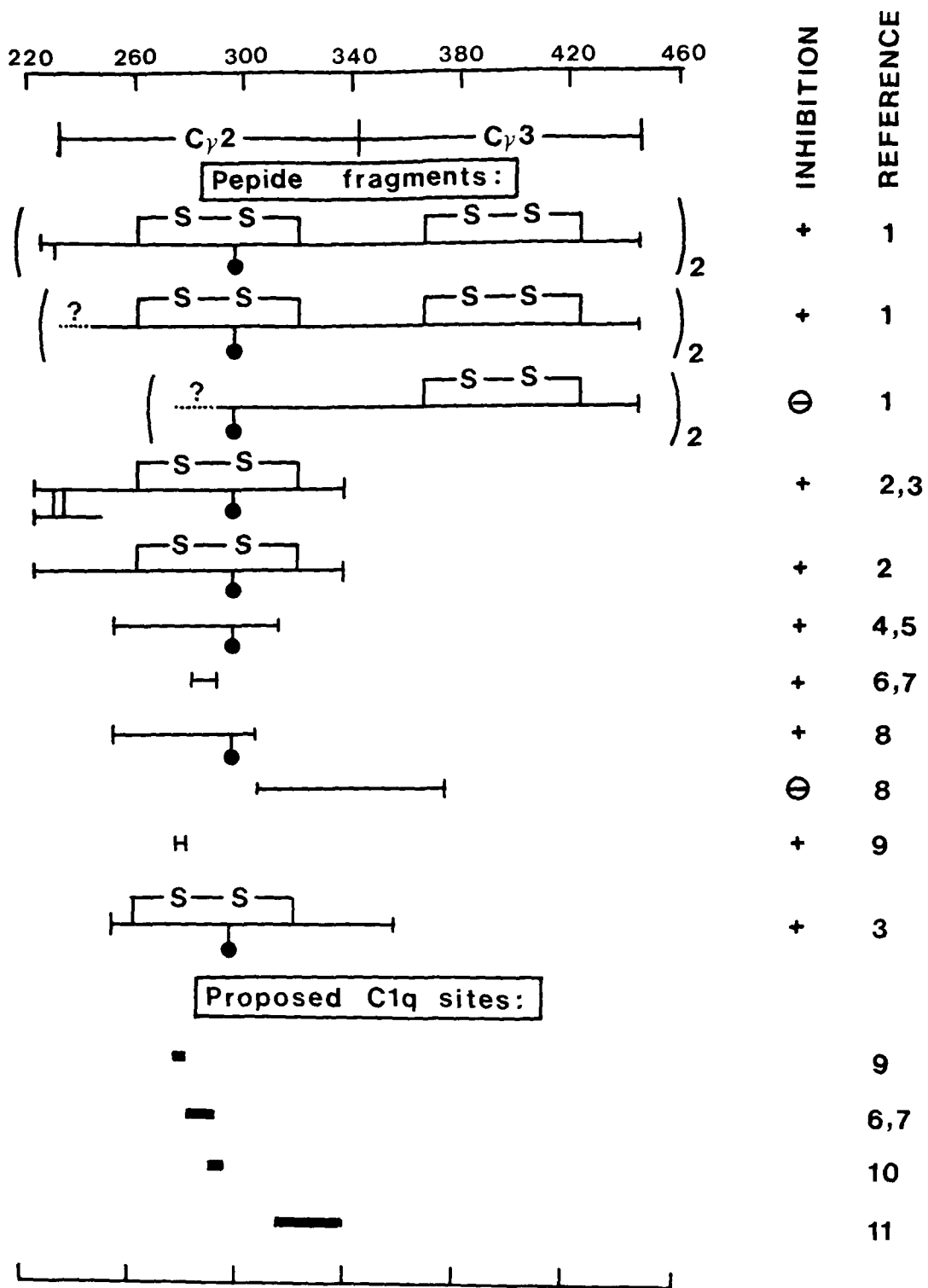


Figure 1.13 Location and inhibitory action of C_γ2 domain fragments in relation to proposed C1q binding sites.

1. Utsumi, 1969
2. Yasmeen *et al.*, 1976
3. Painter *et al.*, 1982
4. Kehoe & Fourgereau, 1969
5. Kehoe *et al.*, 1974
6. Lukas *et al.*, 1981
7. Prystowski *et al.*, 1981
8. Lee & Painter, 1980
9. Boackle *et al.*, 1979
10. Brunhouse & Cebra, 1979
11. Burton *et al.*, 1980

the basis of the marked effect of ionic strength on the IgG-Clq interaction (Lin & Fletcher, 1978; Hughes-Jones, 1977; Burton et al., 1980; Emanuel et al., 1982a); and the inhibition of this interaction by organic diamines (Sledge & Bing, 1973) has often been taken to indicate a cationic site with a near-by hydrophobic region. Modification of Trp-277 has been reported to be responsible for the loss of complement-fixing capacity of tryptophan-modified IgG, although this modification did not affect the binding of Clq (Allan & Isliker, 1974a,b) which could indicate a site which was close to this residue.

Various pieces of evidence have implicated the N-terminal end of the C_γ2 domain. Human IgG4 which has a very short hinge region does not bind Cl. The Fc fragment of IgG4 however binds Cl as well as the Fc fragment of IgG1. This suggests that it is the Fab arms which sterically block a site which must therefore be located somewhere close to the hinge (Isenman et al., 1975). Similarly, prolonged papain digestion of Fc produces a fragment lacking some N-terminal residues and unable to fix complement (Utsumi, 1969). The many studies which have used peptide fragments also seem to concur that the N-terminal half of the C_γ2 domain seems more important than the C-terminal half (Fig. 1.13, and particularly Lee & Painter, 1980):

Sub-fragments of the C_γ2 domain have been extensively used to attempt to localize the Clq site. These studies are summarized in Fig. 1.13, and have led to the proposal of the Clq binding site being located at residues 277-281 (Boackle et al., 1979), or at residues 281-290 (Lukas et al., 1981; Prystowski et al., 1981). In this latter proposal the positively-charged residues His-285, Lys-288, Lys-290 and Arg-292 were posulated to particularly important. The use of small peptide

fragments to define the site is, however, less than definitive as C_γ3 domain derived fragments have been shown to inhibit the Clq-IgG interaction even though unlike the intact C_γ2 domain, the intact C_γ3 domain is inactive (Isenman et al., 1977; Painter et al., 1982). This has led these authors to suggest that the structural requirement for Clq binding may be quite commonplace within the Fc, but that tertiary folding limits its expression to the C_γ2 domain in the native molecule.

Analysis of interspecies / interdomain conservation of amino acid residues has led to the proposal of the Clq binding site being located at residues 290-295 (Brunhouse & Cebra, 1979), and 316-340 (Burton et al., 1980). This latter suggestion has been substantiated by chemical modification studies (Emanuel et al., 1982a,b), although other modification studies may be taken to lend support to the former site. Modification of Glu-318 does not affect the anticomplementarity of human Fc, whereas more extensive modification including that of Glu-293 results in a loss of anticomplementary capacity (Vivanco-Marínez et al., 1980; Bradago et al., 1982).

In the light of the above often conflicting evidence, it must be concluded that although the localization of the site to the C_γ2 domain is well-established, more definitive identification of the residues involved has yet to be obtained.

1.6.5 Activation of complement

Activation of complement occurs after antigen binding. This has led to suggestions that the mechanism could involve an allosteric change in the Fc caused by antigen binding in the Fab region, resulting in Cl

binding / activation. The alternative, and more substantiated hypothesis is that aggregation of antibody alone is sufficient to explain this effect (associative model), with the multivalent Clq (12 or 18 sites for IgG per molecule, Schumaker et al., 1976) binding more tightly to the aggregated IgG by reason of the higher density of IgG sites allowing the possibility of multiple binding (Tschopp et al., 1980a). The evidence relating to this question has been extensively reviewed (Metzger, 1974, 1978) and therefore will not be discussed further here.

With regard to the mechanism of activation of C1, several reports indicate that a conformational change occurs in Clq subsequent to antibody binding, but prior to activation of C1r and C1s (Lin & Fletcher, 1978; Golan et al., 1982; Ziccardi, 1982). It is possible that this conformational change influences the conformation of C1r, leading to its autoactivation in a manner similar to that proposed by Dodds et al. (1978).

IgG of some species (although not mouse) may also activate the complement system via the alternative pathway by activation of C3. In this case it is the Fab region which is involved in the interaction (Reid, 1971). Conflicting reports have suggested that the integrity of the inter-heavy disulphide is essential for activation (Gadd & Reid, 1981); or that it is not essential, with monomeric Fab being equally efficient (Albar et al., 1981). The location of the region on Fab which is responsible has not yet been reported.

1.7 BINDING OF IgG TO MEMBRANE RECEPTORS

1.7.1 Biological significance

Receptors for the Fc portion of IgG (FcR γ) are found on the cell surface of many different cell types including monocytes, macrophages, neutrophils, K cells, and B and T lymphocytes (Perlmann et al., 1972; Anderson & Grey, 1974; Kerbel & Davies, 1974; Dickler, 1976). The role of these receptors in the immune response is at present not clearly defined, however the FcR γ on K-cells have been shown to be directly involved in antibody-dependent cell-mediated cytotoxicity (Perlmann et al., 1972). The receptors on macrophages and monocytes are involved in the phagocytic action of these cells towards antibody-coated particles (Rabinovitch, 1967; Diamond & Scharff, 1980; Diamond & Yelten, 1981). It has been shown that Fc receptor isolated from human B cells (Suzuki et al., 1980), and mouse macrophage FcR γ 2b but not FcR γ 2a (Suzuki et al., 1982) possess both Fc-binding and phospholipase A₂ activity which is stimulated by IgG binding. This enzyme catalyses one of the rate-limiting steps of prostoglandin biosynthesis.

1.7.2 Properties of binding

Of the human IgG subclasses it is found that IgG1 and IgG3 bind to Fc receptors whereas IgG2 and IgG4 bind weakly or not at all (Huber et al., 1971; Haeffner-Cavaillon et al., 1979). The situation in mouse is complicated by subclass-specific receptors (see section 1.7.3).

Although the binding constant varies between cell types, the binding of monomeric IgG to Fc receptor is much tighter than the

Table 1.3

Localization of the cytophillic site on IgG

Region of IgG			
required	not involved	cells used	Reference
Fc		human leukocytes	Quie <u>et al.</u> , 1968
C _H ³	C _H ²	guinea pig macrophages	Yasmeen <u>et al.</u> , 1973, 1976
C _H ³		human monocytes	Okafor <u>et al.</u> , 1974
C _H ²	C _H ³	human lymphocytes	Wisløff <u>et al.</u> , 1974
C _H ³		K cells, neutrophils	MacLennan <u>et al.</u> , 1974
Fc		mouse macrophages	Unkeless & Eisen, 1975
whole Fc	individual C _H ² , C _H ³	human lymphocytes	Michaelsen <u>et al.</u> , 1975
C _H ³		human monocytes	Ciccimara <u>et al.</u> , 1975
C _H ³	C _H ²	mouse lymphocytes	Ramasamy <u>et al.</u> , 1975
C _H ²	C _H ³	guinea pig lung macrophages	Ovary <u>et al.</u> , 1976
whole Fc	individual C _H ² , C _H ³	human granulocytes	Barnett Foster <u>et al.</u> , 1978
whole Fc, intact S-S	individual C _H ² , C _H ³	mouse macrophages	Haeflner-Cavaillon <u>et al.</u> , 1979
C _H ²	C _H ³	mouse macrophages	Diamond <u>et al.</u> , 1979
Fc > C _H ³	C _H ²	human monocytes	Barnett Foster <u>et al.</u> , 1980
a complete hinge		human B cells, neutrophils; mouse macrophage IgG2a receptor	Klein <u>et al.</u> , 1981
	a complete hinge	mouse macrophage IgG2b receptor	" "
correct C _H ¹ region in whole IgG		mouse macrophages	Birshtein <u>et al.</u> , 1982

analogous interaction with Clq. Reported values of the association constant are in the range 10^6 to 10^9 M^{-1} (Unkeless & Eisen, 1975; Kurlander & Batker, 1982). Aggregated IgG usually binds tighter than does monomer (Segal & Hurwitz, 1977), although this depends to some extent on the system studied - the very high affinity human monocyte receptor shows only slightly tighter binding to aggregated IgG, whereas the lesser affinity shown by human neutrophil Fc receptor for monomer IgG (~ 100 - 1000 fold less than that of monocytes) is compensated by a much greater increase in affinity for aggregated IgG, perhaps suggesting 'monomeric-' and 'aggregated-' IgG-specific receptors (Kurlander & Batker, 1982). The Fc receptor responsible for antibody-dependent cell-mediated cytotoxicity seems to only require monomeric IgG (Jones *et al.*, 1979). It has recently been proposed that Clq may also function as an Fc receptor on the cell surface of macrophages during the secretion phase of Clq by these cells (Loos, 1982).

1.7.3 Location of the binding site on IgG

Much experimental work has been undertaken to attempt to localize the domain(s) of IgG which is responsible for binding to cells. The evidence is summarized in Table 1.3. Although it has been found that without exception the site is located in the Fc portion of the molecule, there is a bewildering amount of conflicting evidence as to whether the site is found in the $C_{\gamma}2$ or $C_{\gamma}3$ domains, or requires both. It is probable that this confusion arises to a great extent because of the varying cell lines / types studied (which may possess different receptors), the differing assays used, and the use of non-homologous IgG / cells. This latter point is quite important as unlike the case with Clq, the Fc receptors of different species show significant

differences. In mice it has been clearly demonstrated that macrophages and various macrophage-like cell lines possess three functionally distinct classes of FcR γ : those which bind IgG2b/IgG1 ("FcR γ 2b"), distinguished by resistance to proteolysis by trypsin; those which bind IgG2a (trypsin sensitive); and a less-well characterized IgG3 receptor (also trypsin resistant) (Walker, 1976; Unkeless, 1977, 1979; Heusser *et al.*, 1977; Diamond *et al.*, 1978; Diamond & Scharff, 1980; Diamond & Yelten, 1981). There is some contradiction as to whether the γ 2a and γ 2b receptors are distinct molecular species, or the same molecule in two different states. Anderson & Grey (1978) report that these two receptors are physically distinct species. As noted earlier, confirmation for this has recently come from the fact that as well as differing in charge properties, isolated detergent-solubilized FcR γ 2b is distinguished from a similar FcR γ 2a preparation by possession of phospholipase A₂ activity (Suzuki *et al.*, 1982). Other workers however, have reported that FcR γ 2a and FcR γ 2b lose their subclass discrimination when isolated by detergent solubilization: Mellman & Unkeless (1980) find that the isolated FcR γ 2b (reported to be a glycoprotein and consisting of two diffuse bands of apparent molecular weight 60,000 and 47,000 daltons on SDS-PAGE) binds IgG1, IgG2b and IgG2a (but not IgG3 or F(ab')₂); Schneider *et al.* (1981) report similar observations for both the isolated individual γ 2a and γ 2b receptors, and suggest that they appear to be the same protein, each exhibiting an identical SDS-PAGE profile of a single diffuse band of 50,000 - 65,000 daltons apparent molecular weight.

Whatever its cause, this type of sub-class specific receptor heterogeneity appears to be lacking in humans. However recent work using specific monoclonal antibodies against Fc receptors has shown that

in humans the receptors on neutrophils ($\text{FcR}_{\gamma\text{N}}$) differ from those on monocytes ($\text{FcR}_{\gamma\text{M}}$) both in their antigenic composition and their ability to bind monomeric IgG1 (Fleit et al., 1982). This gives a structural explanation for the observation of large differences in the avidity of neutrophils and monocytes for monomeric IgG1 as discussed in the previous section.

In summary the following may be deduced about the binding site on IgG.

1. It seems likely that different FcR_{γ} may have different structural requirements for binding IgG, therefore an observation made on one system is not necessarily valid for all.
2. Although in individual cases a single isolated domain (either $\text{C}_{\text{H}}2$ or $\text{C}_{\text{H}}3$) binds to the receptor, it is usually not bound as well as either the intact IgG or the Fc fragment.
3. There are reports where a large effect on binding is caused by alterations which might not be expected to perturb a site which was simply located on a single domain. These include: incorrect $\text{C}_{\text{H}}1$ domain (Birshtein et al., 1982); deletion of the hinge region (Klein et al., 1981); incorrect $\text{C}_{\text{H}}2$ / $\text{C}_{\text{H}}3$ domain (Diamond et al., 1979); reduction of interheavy -S-S- in the Fc fragment (Haeffner-Cavillon et al., 1979) (compare this with the lack of any effect of such reduction on Clq binding [Isenman et al., 1975]).

1.8 BINDING TO STAPHYLOCOCCAL PROTEIN A

1.8.1 Interaction of IgG with protein A

Staphylococcal protein A (SpA) is a cell wall component which occurs in many strains of Staphylococcus aureus and is found to interact with most mammalian IgGs (Goding, 1978; Langone, 1982) and also IgA and IgM of certain species and subclasses (Howell-Saxton & Wettstein, 1978, and references therein). The interaction is of high affinity (Kronvall et al., 1970; Lancet et al., 1978) and is specific for the Fc region of the immunoglobulin (Forsgren & Sjöquist, 1966). The SpA molecule comprises five domains. Four are highly homologous and are Fc binding whereas the fifth, C-terminal domain, acts to anchor the protein to the cell wall and does not interact with Fc (Sjödahl, 1977a,b). Partial tryptic digestion produces univalent low molecular weight fragments which retain the binding ability of the parent molecule (Hjelm et al., 1975). One of these fragments (fragment B) has been co-crystallized with human Fc γ_1 and shown to bind at the C γ_2 -C γ_3 domain interface as shown in Fig. 1.6 (Deisenhofer et al., 1978; Deisenhofer, 1981), a position also implicated from work on binding of SpA to IgG fragments (Lancet et al., 1978). The binding of SpA to IgG is therefore the most structurally well characterized of all the IgG secondary interactions.

Due to its unique specificity protein A has been widely used as an immunological tool (see Goding, 1978 and Langone, 1982 for reviews). Its defined binding site on IgG also allows it to be used as a structural probe for the integrity of this site, and it is for this purpose that it is utilized in this thesis (see chapter 6).

1.9 OTHER IgG FUNCTIONS

1.9.1 Control of catabolic rate

When compared with other serum proteins it is noticeable that the half-life of IgG in the circulation is quite large (see Table 1.2). If fragments of IgG are tested it is found that while Fc and C_γ2 domain fragments are cleared at a similar rate to IgG; Fab and pFc' are far more rapidly removed from the circulation (Yasmeen et al., 1976). This suggests that the C_γ2 domain is responsible for controlling the catabolic rate of the intact molecule.

Protein oligosaccharides have been much implicated in control of catabolic rate (see section 6.1.1), and in line with this Winkelhake & Nicolson (1976) have suggested that sialic acid removal from IgG results in rapid clearance. This is contradicted however by other workers who find that sialic acid removal has no effect on clearance rates of IgG (Speigelburg & Weigle, 1966; Yasmeen et al., 1976). This is substantiated by the sequence data on IgG carbohydrate which is found to contain a large proportion of already-exposed terminal galactose residues (Mizuochi et al., 1982; Rademacher et al., 1983) (see section 1.4.3). Subfragments of the C_γ2 domain, including glycosylated peptides, do not exhibit the long half-life of the intact domain (Lee & Painter, 1980). This suggests that it is some tertiary protein structure which determines the catabolic rate.

Exposure of terminal galactose residues upon immune complex formation has been suggested as a mechanism for clearance of IgG/antigen complexes by hepatocytes (Thornberg et al., 1980). Immune complex

formation alone is insufficient however, as such complexes in isolation do not bind to isolated hepatocytes (Baenziger & Fiete, 1980). The results of Thornberg et al. (1980) could be explained if some additional serum factor was necessary. In this respect it is interesting that binding of Clq (but not binding of antigen) induces a change in the environment of the oligosaccharide on IgG, as sensed by alterations in the mobility of a nitroxide label specifically attached to sialic acids of the N-linked sugar chain of rabbit IgG (M. M. Beale & R. A. Dwek, unpublished observations).

1.9.2 Materno-foetal transfer

In mammals, passive transfer of immunity is conferred to offspring by transmission of maternal immunoglobulin which is exclusively of the IgG class (Stanworth & Turner, 1978). This usually occurs at either a pre-natal (as in man) or a post-natal stage, but may occur at both (as in mouse). Pre-natal transfer occurs via the placenta in man and the yolk sac membrane in rabbits; post-natal transfer is across the gut wall of newborn rodents. All subclasses of IgG appear to be transferred, although slight variations in receptor affinity are found which results in slightly different subclass proportions in the maternal and foetal circulation (Guyer et al., 1976). It has been shown that it is again the Fc region which is responsible (Stanworth & Turner, 1978; Tsay & Schlamowitz, 1978). In rabbits the site appears to be located on a single C_H2 domain (Johanson et al., 1981), however in mouse neither C_H2 nor C_H3 fragments were found to have the activity of intact Fc (Guyer et al., 1976).

1.9.3 Passive cutaneous anaphylaxis

IgG of certain subclasses possesses skin-sensitizing activity (Brocklehurst, 1978). The PCA reaction is due to degranulation of mast cells and the local release of vaso-active amines mediated by IgG binding to receptors on these cells and then interacting with antigen. The region of IgG responsible for cell binding is Fc, and the PCA reaction is abolished by loss of the interheavy disulphide (Utsumi, 1969). Although IgG does bind to mast cells, it is not as tightly bound as IgE, and therefore much larger concentrations are required to generate a comparable response.

1.9.4 B cell activation

Much recent work by Morgan & Weigle has shown that Fc fragments of IgG will, in the presence of macrophages and T cells, stimulate polyclonal activation of B lymphocytes in both human and mouse systems. This is dependent upon macrophage cleavage of Fc (as such or in the form of immune complexes) to produce subfragments of 14,000 M_r which interact with T cells to generate a differentiative signal to the B cells (see Morgan et al., 1982 and references cited therein). The 14,000 M_r subfragment is derived from the C_H3 domain, and activity is totally accounted for by a 24 amino acid peptide comprising residues 335-358 (Morgan et al., 1982).

1.10 MOPC 315 - A BRIEF REVIEW

1.10.1 The properties of protein 315

In a study by Eisen et al. (1968) to screen transplantable mouse plasma cell tumours for antigenic activity, out of 116 tumours seven showed anti-dinitrophenyl activity. The most active of these was found to be the protein produced by tumour MOPC-315. The immunoglobulin produced by this plasmacytoma is of the IgA class and contains the unusual λ_2 light chain subclass, found in only 1% of immunoglobulins isolated from normal mouse serum (Blaser & Eisen, 1978). It is possibly this feature which accounts for the unusual pepsin cleavage of protein 315 which generates in high yield a fragment (Fv) containing only the variable domains of the molecule (Inbar et al., 1972). This fragment retains the combining site structure of the native protein (Inbar et al., 1973; Morris et al., 1978).

Protein 315 shows remarkable similarities to naturally induced anti-DNP antibodies: it has a comparably high affinity for DNP haptens ($K = 1.6 \times 10^7 \text{ M}^{-1}$ for DNP-lysine); bound ligands undergo a characteristic red shift (Little & Eisen, 1967) and quench the protein tryptophan fluorescence; and precipitates are formed on addition of dinitrophenylated proteins. Two equivalent homogeneous sites are found per molecule, which are located on the two Fab regions (Underdown et al., 1971). Therefore protein 315 provides an excellent system for studies on the properties of antibody combining sites. This has resulted in a large number of such studies on this protein, which have provided much information on the nature of its interaction with haptens.

Table 1.4

Chemical modification of residues in or near to the combining site of protein 315

Residue	Modifying reagent	Effect on hapten binding	Ref
Tyr-34 _L	m-nitrobenzene diazonium fluoroborate (MNBD)	Fifty-fold reduction	1
Tyr-34 _L	N-(bromoacetyl)N'-(dinitro- phenyl)-ethylenediamine (BADE)	No binding	2
Tyr-34 _L	Tetranitromethane (TNM)	No effect	3
Lys-52 _H	α bromoacetyl- ϵ ,N-DNP-L-lysine (BADL)	No binding	2
Tyr-33 _H	TNM	No effect	4
"tyrosines"	ICl	Twenty-fold reduction	5
"arginines"	Glyoxal	Reduced no. of sites	6
"lysines"	Maleic anhydride	No effect	7

References:

1. Goetzl & Metzger , 1970a,b
2. Haimovich et al., 1970, 1972; Givol et al., 1971
3. Gavish et al., 1979
4. Dwek et al., 1977; Wain-Hobson, 1977
5. Klostergaard et al., 1978
6. Klostergaard et al., 1977b
7. Klostergaard et al., 1977a

1.10.2 Features of the combining site of protein 315

A summary of the available evidence on the structural features of the combining site of this protein is given below.

Involvement of a tryptophan residue As described above, DNP and TNP haptens when bound to protein 315 undergo a red-shift in their visible region spectral absorption. This is suggestive of a stacking interaction with the side chain of tryptophan residue in the combining site (Little & Eisen, 1967). This conclusion is supported by studies monitoring changes in the CD spectrum on hapten binding (Freed et al., 1976; Orin et al., 1976). Since a similar (although weaker) interaction is maintained by the V_L and light chain dimers it seems that the relevant tryptophan is contributed by the light chain (Schechter et al., 1976; Freed et al., 1976; Gavish et al., 1977, 1978).

Chemical modification Studies in which residues in or near to the combining site have modified either by group-specific or affinity labels are listed in Table 1.4. From these studies it is evident that Tyr-34_L and Lys-52_H must be either in the site, or close proximity to it.

Interactions with hapten side chains By monitoring the interaction of protein 315 with a range of different DNP haptens it was found that a significant contribution to the overall binding was made by interactions with the side chains of these ligands (Haselkorn et al., 1974). As well as the DNP subsite, two hydrophobic and a peripheral positively-charged subsite were inferred. By comparison of the binding affinities of a similar range of haptens to the V_L dimer it seems likely that a significant proportion of the side chain interactions are with

heavy-chain residues (Gavish et al., 1977).

Hydrogen bonds to the ligand nitro groups The presence of such interactions has been determined by substitution of $-\text{NO}_2$ by groupings of similar electron-withdrawing ability, but which lack the acceptor properties necessary to form hydrogen bonds. Substitution with $-\text{CF}_3$ has led to the conclusion that hydrogen bonds are formed to both the 2- and 4-nitro groups, but that the 2-nitro group is more important in this respect (Hardy & Richards, 1978). Substitution with $-\text{Cl}$ confirms the importance of these protein-nitro group hydrogen bonds, the results indicating approximately equal contributions to each nitro group (Gettins et al., 1978). Direct observation of nitro group stretching modes by resonance Raman spectroscopy also demonstrates specific interactions of both the 2- and 4-nitro groups with the protein (Gettins et al., 1981). However with one hapten, DNP-lysine, the interaction with the 4-nitro group is absent suggesting that such hydrogen bonding may not be necessary for all haptens.

Overall dimensions By use of a series of differing spin-labelled DNP haptens, Sutton et al. (1977) were able to determine the following overall dimensions. The depth of the site is 1.1-1.2 nm with an entrance which is assymmetric with respect to the plane of the DNP ring. A positively-charged residue is found on the more restricted side of the site, close to the entrance.

1.10.3 Postulated combining site models of protein 315

As described in section 1.3.1 the variable domains of immunoglobulins may be described in terms of a fixed framework onto

Table 1.5

Hypervariable loop structures used by Padlan et al. (1976) as a basis for their predicted structure of the combining site of protein 315. The framework variable region used was that of McPC 603 Fab'.

Loop	Residues involved	Conformation based on:	Comments
L1	23-36	Mcg ^a	Same length as 315, both are λ
L2	52-58	McPC 603 ^b	Same length as 315. REI ^c and Mcg are also the same length and all have similar conformations
L3	91-99	McPC 603	315 has the potential for more H-bonds in this loop - this was incorporated
H1	31-36	McPC 603	Extra residue in 315 accomodated, basic 603 structure retained
H2	49-66	McPC 603/New ^d	Three extra residues in 603 excised, then loop adjusted to resemble New
H3	99-104	McPC 603/New	Two extra residues in 603 excised from C-terminal end, then made to resemble New except for C-terminal end

^a Schiffer et al., 1973

^b Padlan et al., 1973; Segal et al., 1974

^c Epp et al., 1974 (also see Epp et al., 1975)

^d Poljak et al., 1974 (also see Saul et al., 1978)

which the hypervariable regions are attached. Given the sequence of these regions, and knowing the locations of the attachment points, it is possible to construct a hypothetical model of the hypervariable region structure. This may then be useful in explaining the structural features determined from other studies.

On the basis that hypervariable loops of similar length are likely to have similar structures, Padlan et al. (1976) constructed a model of the combining site of protein 315. The criteria used to determine the construction are given in Table 1.5, and the resultant site in Fig. 1.14. The structure that emerged showed a cleft between the heavy and light chains and containing the side chain of Trp-93_L. An interaction of haptens with this tryptophan side chain would place their side chains in an appropriate position to account for affinity labelling of Tyr-34_L and Lys-52_H, which were located around this proposed binding site cleft.

The plausibility of this proposed site prompted Dwek and co-workers to use it as a starting point for further refinement. The locations of the residues in this site were altered to be consistent with physical studies, mainly involving use of NMR (see reviews by Dwek, 1977 and Dower & Dwek, 1979). The basis of this is as follows. ¹H-NMR difference spectra produced on binding of DNP haptens to the Fv fragment of protein 315 show minimal differences with only 2-3% of resonances perturbed (Dower et al., 1977). Of these the majority are resonances of aromatic protons. This is interpreted as binding causing only very localized perturbations to a predominantly aromatic binding site. The DNP ring protons are found to experience large upfield shifts (1-2.5 ppm) on binding to the protein. In consideration of the aromatic

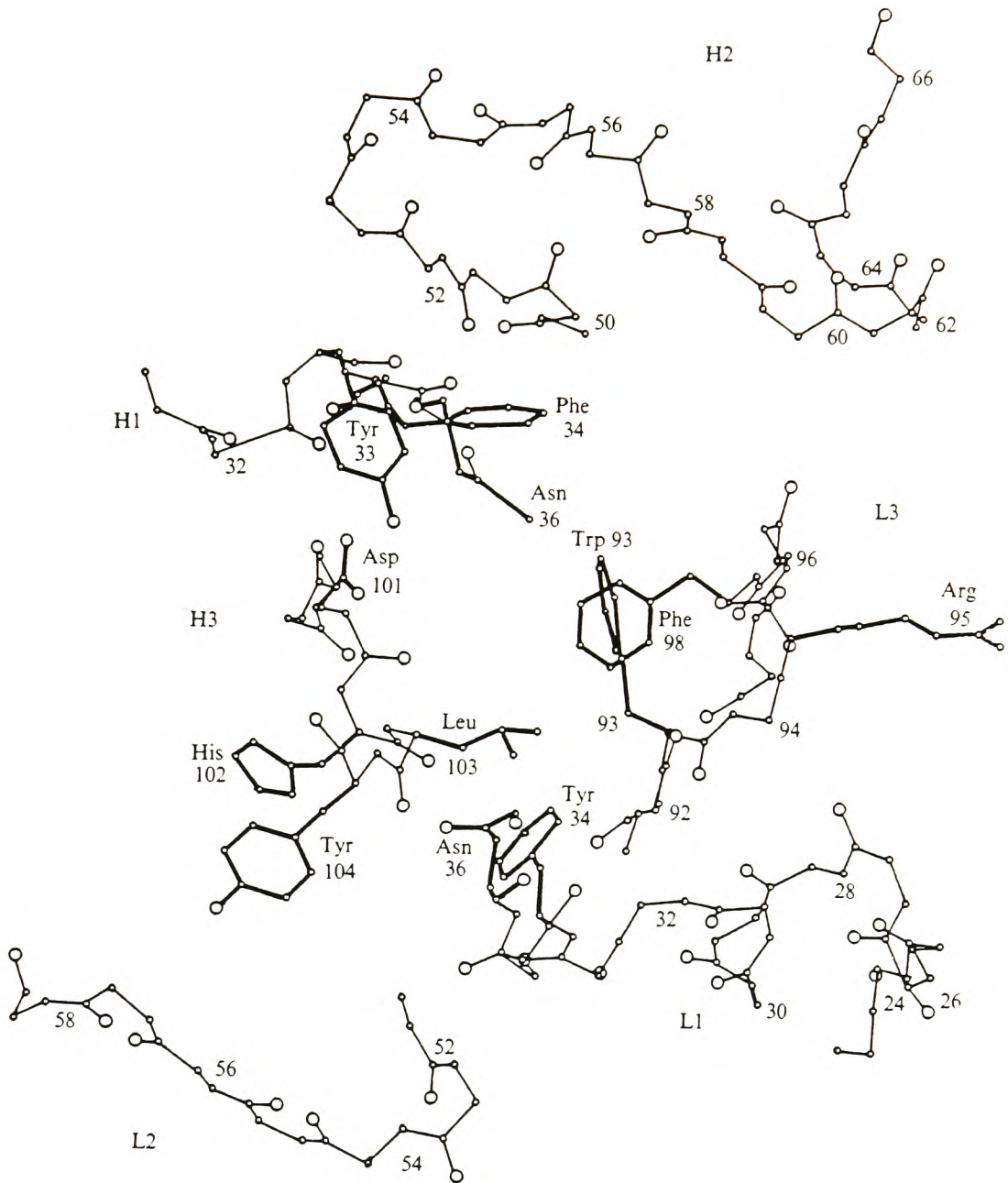


Figure 1.14 The combining site of protein 315 as built by Padlan et al. (1976).

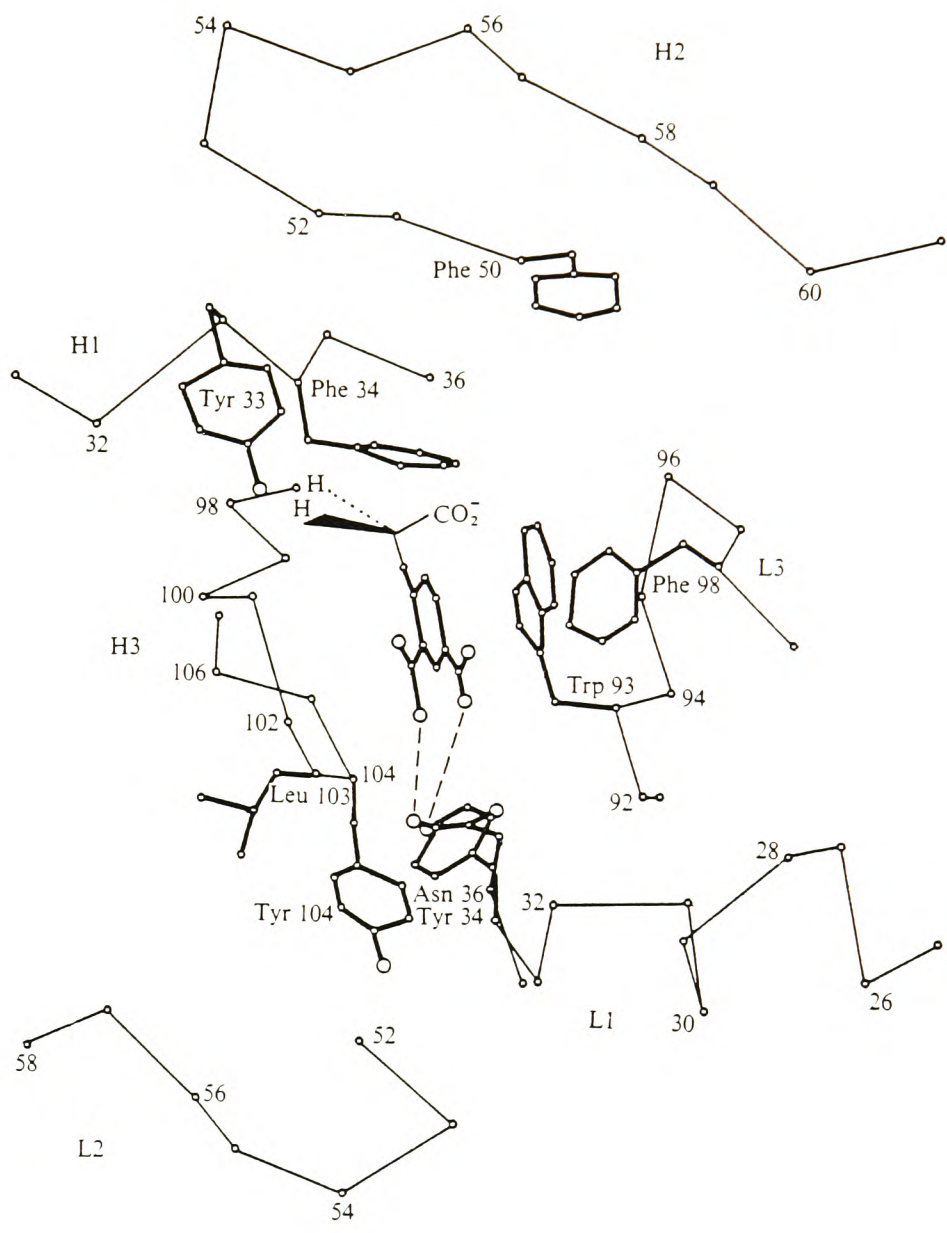


Figure 1.15 The combining site of protein 315 as described by Dower et al. (1977).

nature of the binding site, these are interpreted as arising mainly from ring-currents produced by neighbouring aromatic residues. Since it is possible to calculate the theoretical magnitude and geometric dependence of such shifts (Perkins et al., 1977, also see appendix 2) they may be used as a filter to screen for feasible orientations of the hapten with respect to the surrounding aromatic side chains. The factors and assumptions involved in such calculations are considered further in chapter 3. The resultant site described by Dower et al. (1977) is shown in Fig. 1.15. The main features of this site are:

1. The DNP ring is in association with the side chain of Trp-93_L.
2. Surrounding aromatic residues which are responsible for the large chemical shift changes on the bound hapten ring protons are Trp-93_L, Phe-34_H and Tyr-34_L.
3. The side chain of Tyr-33_H is in the entrance of the site, and contributes to the change in chemical shift of hapten side chains.
4. The positively-charged residue in the combining site entrance was suggested to be Arg-95_L.
5. Hydrogen bonds are formed to the 2-nitro group from the side chain of Tyr-34_L, and to the 4-nitro group from the side chain of Asn-36_L.

1.11 THE CURRENT INVESTIGATION

1.11.1 The combining site of protein 315

While there has been much previous work on the structure of the combining site of protein 315, there are reasons for re-examination of the predicted sites. Firstly, recent work has suggested that the positioning of Tyr-34_L to form a hydrogen bond to the 2-nitro group of DNP ligands is incorrect (Gavish et al., 1979). Yet as discussed in section 1.10.2 there is much evidence suggestive of some hydrogen bonding interaction to this nitro group. Secondly, from a detailed analysis of the applicability of ring-current calculations to predict experimental chemical shift changes in proteins (Perkins & Dwek, 1980) it may be inferred that the analysis used by Dower et al. (1977) could have considerably underestimated the chemical shifts produced by the surrounding aromatic residues in the site. Chapter 2 investigates the location of Tyr-34_L and Tyr-33_H in the combining site by use of specifically nitrated derivatives of the Fv fragment. In chapter 3 the effect of the revised ring-current intensity factors of Perkins & Dwek (1980) on structural predictions of the combining site are examined in the light of the results of chapter 2.

1.11.2 The role of the N-linked oligosaccharide of IgG

Immunoglobulin G is presently the most well-characterized glycoprotein. Of four crystal structures of glycoproteins which are available one is of the hinge-deleted IgG Dob (Silverton et al., 1977) and two are of the Fc fragment of IgG (Deisenhofer, 1981; Sutton & Phillips, 1983) (the remaining structure is that of influenza virus

haemagglutinin (Wilson et al., 1981). In spite of this the involvement of the oligosaccharide in the structure / function of IgG is poorly understood.

The remaining chapters of this thesis attempt to clarify the role of this conserved N-linked oligosaccharide by a functional comparison of normal glycosylated IgG with aglycosyl IgG produced in cell culture in the presence of the glycosylation inhibitor tunicamycin. In chapter 5 the conditions for the production of aglycosyl IgG in cell culture are determined, and the preparation of aglycosyl IgG in sufficient quantities and purity for functional studies is described. Chapter 6 investigates the functional characteristics of this aglycosyl IgG in terms of Clq binding, Cl activation, cell binding, protein A binding, and proteolytic susceptibility. For the Clq binding and Cl activation an assay system was required which allows a quantitative comparison of immunoglobulins which may have different immune-complex lattice characteristics. The development of an assay suitable for this purpose is described in chapter 4. Finally in chapter 6 from an analysis of the quaternary arrangement of the Fc domains some indications of the structural effects and role of glycosylation in IgG are obtained.

CHAPTER 2

The Role of Tyrosines in the Combining Site of Protein M315: Specific Nitration and High Resolution Hydrogen-1 Nuclear Magnetic Resonance Studies

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2.1 INTRODUCTION

2.1.1 Chemical modification of antibody combining sites

Specific chemical modification of amino acid side chains is a potentially powerful method of determining their involvement in protein function. With antibodies, affinity labelling has proven useful in determining the proximity of certain residues to the combining site, and the involvement of both L and H chain residues in the site (reviewed by Givol, 1979). Many studies on protein 315 (see section 1.10) have indicated that certain amino acid side chains in or near to its combining site may be modified, with varying effects on binding ability (Table 1.4).

In this chapter, the involvement of Tyr-33_H and Tyr-34_L in the combining site of protein 315 is investigated by a study of suitably nitrated Fv fragments. Nitration of tyrosines by tetranitromethane (TNM) is a convenient method of modification for several reasons: the reaction conditions are mild and non-denaturing; the modifying group is small, minimizing the possibility of large structural perturbations; nitration lowers the pKa of the phenolic group from 10.1 to 7.2 (in model compounds), allowing the effect of its ionization to be observed over a more physiological pH range; the ionization of the phenolic group itself may be monitored spectrophotometrically, enabling its pKa to be determined; and finally the nitrotyrosine may be reduced to aminotyrosine, to provide an attachment point for other spectrophotometric probes if required (Riordan et al., 1966; Sokolovsky et al., 1966; Riordan et al., 1967; Sokolovsky et al., 1967).

2.1.2 The involvement of tyrosines in the combining site of M315

Modification of the Fv fragment with TNM to the extent of one $\text{NO}_2\text{-Tyr mol}^{-1}$ gives 80% $\text{NO}_2\text{-Tyr-33}_\text{H}$ and 20% $\text{NO}_2\text{-Tyr-34}_\text{L}$ (R. Zakut & D. Givol, personal communication). Nitration of the isolated V_L dimer gives exclusively $\text{NO}_2\text{-Tyr-34}_\text{L}$ which can be recombined with V_H to form $\text{NO}_2\text{-Tyr-34}_\text{L}$ Fv (Gavish et al., 1979). The involvement of these tyrosines in the combining site of protein 315 is implicated by:

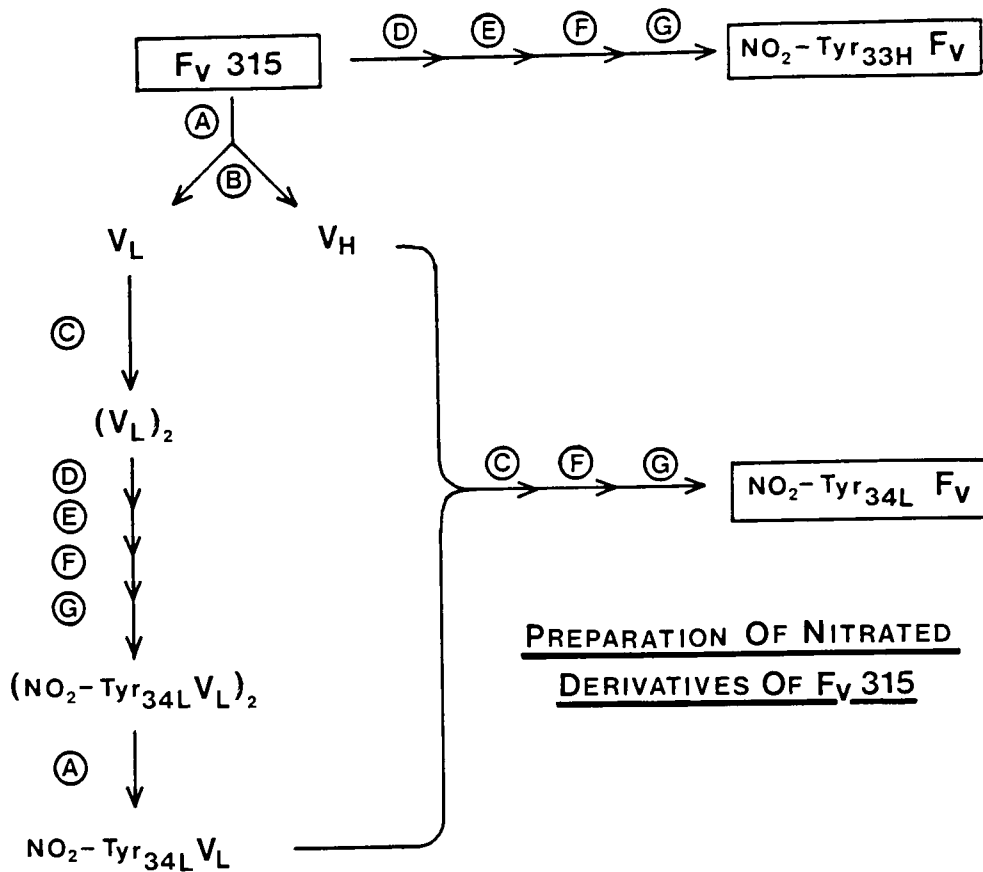
1. Affinity labelling of Tyr-34_L with m-nitrobenzenediazonium-fluoroborate (MNDB), (Goetzl & Metzger, 1970a,b)
2. Affinity labelling of Tyr-34_L with N-(bromoacetyl)N'-(dinitrophenyl)-ethylenediamine (Haimovich et al., 1970, 1972) and other bromoacetyl-DNP derivatives (Givol et al., 1971)
3. Iodination of tyrosines (Roholt et al., 1974; Klostergaard et al., 1978)
4. High resolution NMR studies in conjunction with model-building (Dower et al., 1977; Dwek et al., 1977). These studies positioned Tyr-33_H in close proximity to the hapten side chains, and Tyr-34_L in contact with, and hydrogen-bonded to the 2-nitro group of, the DNP ring.

Affinity-labelling of this tyrosine has also been reported in other antibodies (Franěk, 1971; Chesbro & Metzger, 1972). Since a tyrosine occurs at this position in 50% of all known light chain sequences (127 out of 245, Kabat et al., 1979) it is probable that it plays an important role in many antibody combining sites. Although the only Fab fragment structures which are known do not contain this residue, it occurs in the Bence-Jones proteins REI and Mcg for which X-ray crystallographic data is available (Epp et al., 1974, 1975; Schiffer et al., 1973; Edmundson et al., 1974). In Mcg this tyrosine is

a contact residue for dinitrophenyl ligands (Firca et al., 1978). The proposed role for this tyrosine of hydrogen-bonding to the DNP 2-nitro group (Dower et al., 1977) has recently been found to be inconsistent with the lack of any effect on the binding of ligands to NO₂-Tyr-34_L Fv with pH (Gavish et al., 1979). This places the exact location and role of this tyrosine in doubt.

A tyrosyl residue is also frequently found at position 33_H (27 out of 94 sequences, Kabat et al., 1979). Tyr-33_H is known to be a contact residue in protein M603 (see section 3.3.1 and Fig. 3.2), where its phenolic group hydrogen bonds to the phosphate group of the phosphocholine ligand. A similar hydrogen bonding scheme to DNP ligands in protein 315 does not occur (Dwek et al., 1977; Wain-Hobson, 1977). However, NMR studies in conjunction with model-building have suggested that the aromatic ring of this residue is proximal to the side chain of DNP haptens when bound to this protein. A heavy-chain tyrosine has also been implicated in the combining site of protein 315 by modification-protection experiments (Roholt et al., 1974).

In view of the probable importance of these two tyrosines in protein 315, the work in this chapter was undertaken to provide further information on their location relative to the hapten binding site, and therefore give some insight as to their possible role in this site.



- (A) Dissociation in 8M Urea
- (B) DEAE-Cellulose Chromatography in 8M Urea
- (C) Dialysis versus buffer
- (D) Nitration with TNM
- (E) Sephadex G-25 Chromatography - Separates Protein from Reactants
- (F) DNPL-Sepharose Affinity Chromatography - Active Protein eluted with DNP-glycine
- (G) Dowex 1X8-400 Chromatography - Removes DNP-glycine

Figure 2.1 Schematic illustration of the preparation of $\text{NO}_2\text{-Tyr-33}_H \text{ Fv}$ and $\text{NO}_2\text{-Tyr-34}_L \text{ Fv}$.

2.2 MATERIALS AND METHODS

2.2.1 Transplantation of tumour

Plasmacytoma MOPC 315 (Eisen et al., 1968) was grown in F₁ Balb/c x DBA mice as an ascitic tumour as described in section 4.2.2.

2.2.2 Preparation of Fv 315

Monomer IgA M315 was isolated from ascites of tumour-bearing mice by ammonium sulphate precipitation followed by mild reduction / alkylation, and affinity chromatography on DNP-lysine-Sepharose (Inbar et al., 1971). Fv was prepared by peptic digestion of this IgA (Hochman et al., 1973): IgA was digested for 6 hours at 37°C with pepsin (1:100 w/w) at pH 4.5, followed by further addition of 1:100 pepsin and incubation for 4 hours at pH 3.8. The digestion was terminated by raising the pH to 7. Fv was isolated by Sephadex G-75 gel permeation chromatography of the concentrated products (Inbar et al., 1972). The purity of the product was verified by SDS-PAGE (Laemmli, 1970), and its activity by fluorescence quenching titrations (Eisen, 1964b; Eisen et al., 1968).

2.2.3 Preparation of nitrated Fv derivatives

The preparation of these derivatives is shown schematically in Fig. 2.1.

Nitration of Tyr-33_H: This was performed by reacting Fv fragment (4×10^{-5} M) in 0.1 M tris-HCl pH 8.2, with TNM (2.2×10^{-4} M, added in ethanol such that the final concentration of ethanol was 1%) for 1 hour

at room temperature (Sokolovsky et al., 1966; Wain-Hobson, 1977). The reaction was stopped by separating protein from reactants on a Sephadex G-25 column in 0.1 M $(\text{NH}_4)_2\text{SO}_4$. Active Fv was isolated on a DNP-lysine-Sepharose affinity column and eluted with 50 mM DNP-glycine (Hochman et al., 1973). Approximately 40% of the material did not bind to the column. The active material was found to be indistinguishable from native Fv fragment when analysed by SDS-PAGE, whereas the inactive material clearly contained covalent aggregates (results not shown), as is frequently observed in nitration of tyrosines by this method (Doyle et al., 1968; Boesel & Carpenter, 1970; Vincent et al., 1970). These conditions gave a modification of 1.0 mol of nitrotyrosine per mol of Fv fragment, assuming $E_{428}^M = 4100$ for nitrotyrosine at pH 10 (Sokolovsky et al., 1966).

Nitration of Tyr-34_L: The constituent V_L and V_H domains of Fv were prepared as described by Hochman et al. (1973) using ion-exchange chromatography on DEAE-cellulose (Whatman DE-52) in 8 M urea (deionized on Zerolit DM-F mixed bed resin (BDH) immediately prior to use). The isolated V_L dimer was nitrated by incubation of $(V_L)_2$ (5×10^{-5} M) in 0.1 M tris-HCl pH 8.2 with TNM (5×10^{-4} M) for 1 hour at room temperature (Gavish et al., 1979), and the reaction terminated by passing the mixture through a Sephadex G-25 column as described above. Active $(V_L)_2$ was isolated on a DNP-lysine-Sepharose affinity column and eluted with 50 mM DNP-glycine (Gavish et al., 1977). Recombination of NO_2 - V_L with V_H was achieved by incubation of equimolar quantities for 24 hours at 4°C, and active product (~ 80% of the starting material) was isolated on a DNP-lysine-Sepharose column (Hochman et al., 1973). These conditions were found to give 0.9 mol of nitrotyrosine per mol of V_L domain, which is located exclusively at Tyr-34_L (Gavish et al., 1979).

Irradiation of the desired resonance was performed, and 32 scans were accumulated with an acquisition time of 2.05 seconds plus a delay time of 1 second. Irradiation was then switched to a point outside the spectrum and another 32 scans were accumulated, which were subtracted from the first set. In this manner an NOE-difference spectrum is obtained. This cycle was repeated 140 times to provide adequate spectra.

To increase the signal to noise ratio, all spectra were convoluted using an exponential function which added 2 Hz to the linewidth of the resonances. Chemical shifts are reported as parts per million (ppm) from the methyl resonance of DSS (2,2 dimethylsilapentane- sulphonate [sodium salt]), used as external standard in $^2\text{H}_2\text{O}$. All spectra were recorded using a probe temperature of 303K, maintained using an air-flow temperature controller. An air flow was also used to spin the sample.

2.2.6 Calculation of fully-bound chemical shifts

The binding of hapten to antibody is sufficiently tight that $[\text{hapten}]$ and $[\text{protein}] \gg K_d$. Therefore when $[\text{hapten}]_{\text{total}} > [\text{protein}]_{\text{total}}$ then $[\text{hapten}]_{\text{bound}} = [\text{protein}]_{\text{total}}$. Thus:

$$\frac{\delta_o - \delta_{\text{obs}}}{\Delta\delta_{\text{fully-bound}}} = \frac{[\text{protein}]_{\text{total}}}{[\text{hapten}]_{\text{total}}}$$

and a plot of $[\text{protein}]_{\text{total}}/[\text{hapten}]_{\text{total}}$ against δ_{obs} will yield the fully-bound chemical shift change as $-1/\text{gradient}$.

2.2.7 Absorbance measurements

The absorbance measurements for the pH titrations were made on a Gilford model 250 spectrophotometer. Protein concentration was 25 μM ;

haptens when present were in a slight molar excess. The protein was dissolved in 0.15 M NaCl/0.01 M phosphate pH 8.3 and the pH lowered by sequential addition of 0.12 M HCl/0.15 M NaCl. Measurement of pH was performed directly in the cuvette using a glass microelectrode, and absorbance was corrected for dilution during the titration. The experimental data were fitted to a one-proton titration curve by the FORTRAN 77 program PKA (Appendix 1).

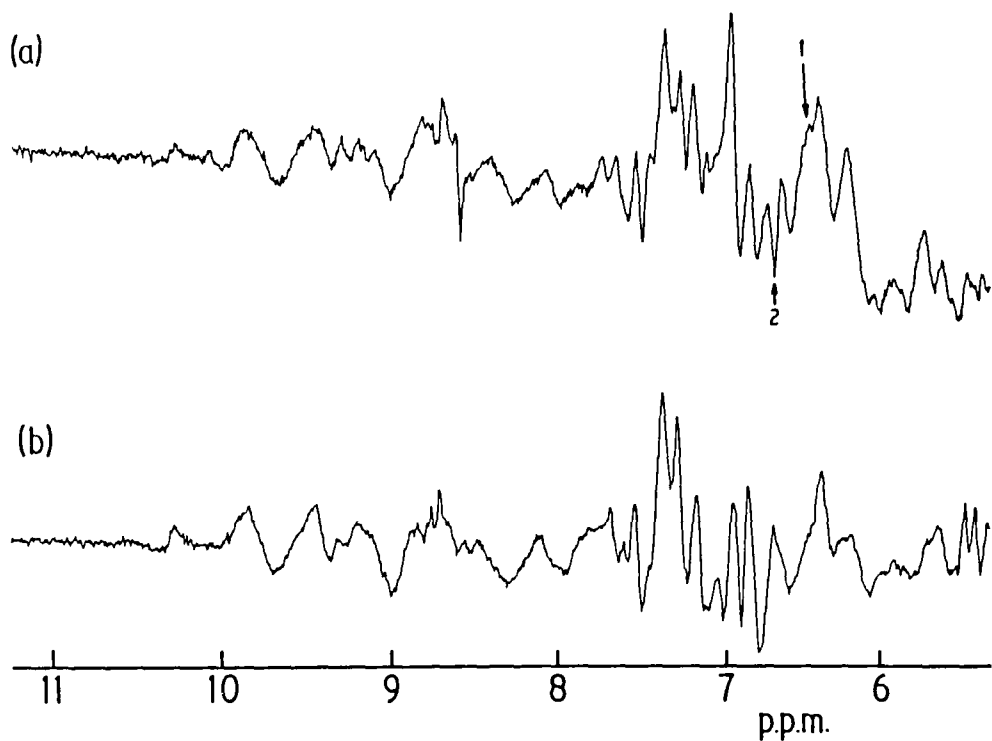


Figure 2.2 470 MHz NMR hapten difference spectra of (a) Fv and (b) NO₂-Tyr-34_L Fv. The hapten (DNP-glycine) is present in a slight molar excess. Conditions are T = 30^oC, protein concentration = 1 mM with 0.15 M NaCl, pH* = 5.0, 1000 scans were accumulated per spectrum. The spectra are essentially identical apart from the absence in (b) of the resonance which titrates from position 1 to position 2 [indicated in (a)] on addition of hapten.

2.3 RESULTS AND DISCUSSION

2.3.1 ¹H NMR studies

Specific chemical modification is a potential method of assigning resonances in the ¹H NMR spectra of proteins. The aromatic proton resonances of nitrotyrosine occur at different field from the parent tyrosine, and titrate upfield with increasing pH thus allowing assignments and determination of pKa values (Snyder et al., 1975; Schmidt-Aderjan et al., 1979; Ribeirto et al., 1981). However, for both the nitrotyrosine Fv derivatives no resonances, other than those of the three histidine C-2(H) and C-4(H) protons, were observed to titrate over the pH range 5-8. This indicates that the NO₂-Tyr Fv resonances are too broad to observe.

Another approach which may result in assignments for these tyrosines is a comparison, between the native and nitrated proteins, of the positions of peaks in the difference spectra produced on binding of hapten. Both nitrated Fv fragments and the native Fv were titrated with DNP-glycine at 470 MHz and at constant pH as described by Dower et al. (1977). The difference spectra from NO₂-Tyr-34_L Fv were found to be almost identical to those produced by native Fv (Fig. 2.2), indicating that nitration causes no significant change in the mode of ligand binding or in the combining site. However a protein resonance in the Fv fragment, which titrates from 6.40 to 6.74 ppm in the presence of the hapten DNP-glycine (arrowed in Fig. 2.2), is absent in the difference spectrum of the NO₂-Tyr-34_L Fv fragment. This resonance can thus be tentatively assigned to Tyr-34_L. At 270 MHz, a possible assignment of a resonance to Tyr-34_L at ca. 6.2 ppm was made on the basis of the effects

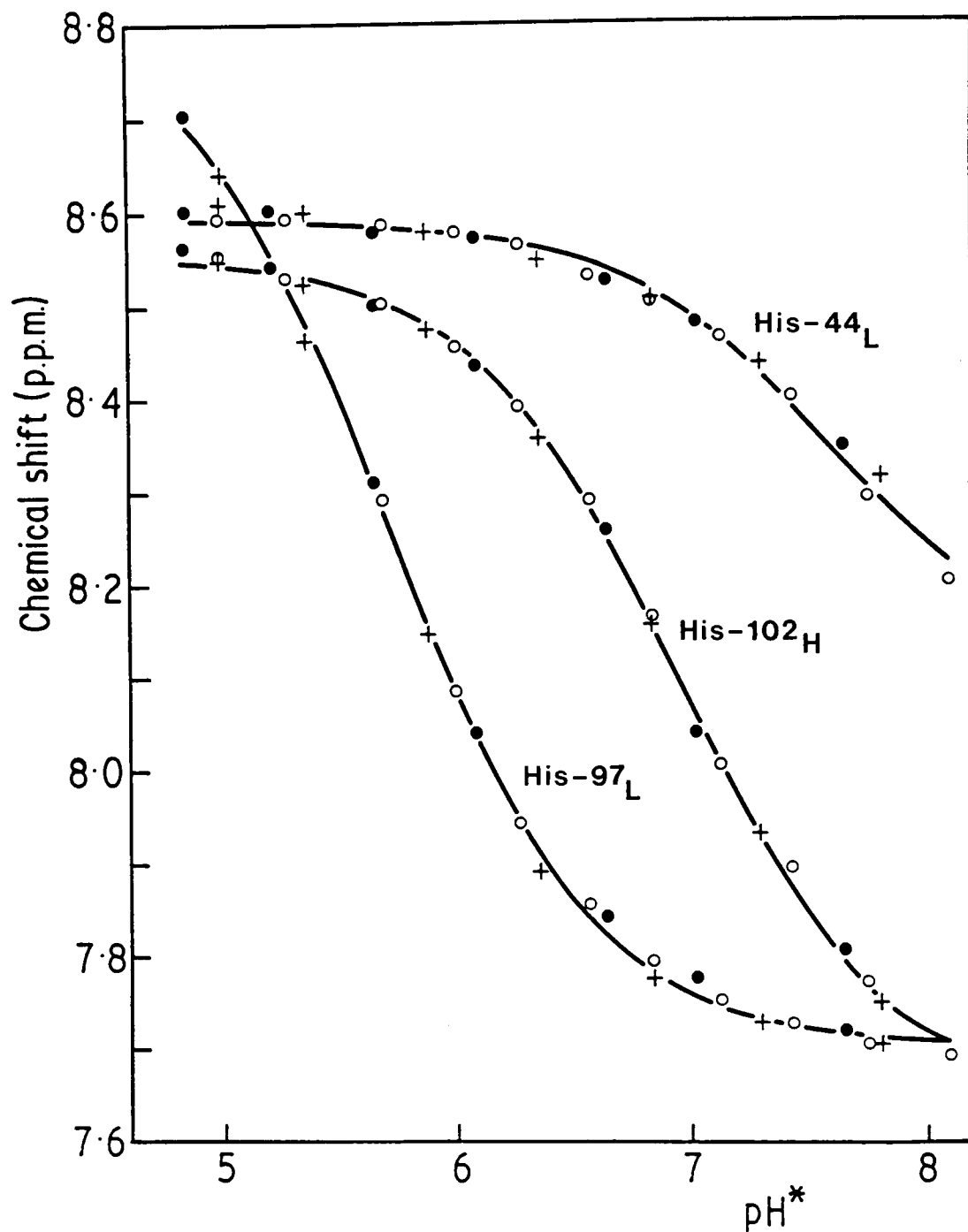


Figure 2.3 pH^* titration of histidine C-2(H) proton resonances at 470 MHz of native Fv (●), $\text{NO}_2\text{-Tyr-33}_\text{H}$ Fv (○), and $\text{NO}_2\text{-Tyr-34}_\text{L}$ Fv (+). Conditions are $T = 30^\circ\text{C}$, protein concentration = 1 mM with 0.15 M NaCl, 1000 scans were accumulated per spectrum. Positions of titrating peaks were obtained from serial difference spectra. Assignments of histidine C-2(H) resonances are from Wain-Hobson *et al.* (1977). The solid curves represent the best fit to the data assuming a single proton titration curve.

of guanidine hydrochloride on the Fv spectrum (Gettins & Dwek, 1981). However, at 470 MHz, there is a marked increase in resolution of the broad spectral region between 6.2 and 6.6 ppm, splitting out more individual resonances which can then be more accurately followed in titrations.

Whatever the assignment problems, the ^1H NMR difference spectra of native minus nitrated protein can be used to measure the extent of the structural perturbations caused by nitration. These difference spectra contain only about 1-2% of the original spectral intensity, emphasizing that modification of the tyrosines causes little or no perturbation of the protein structure beyond the immediate environment of the nitro group.

The pH titration behaviour of the three histidine resonances provides further evidence that any perturbations are very localized. As is shown in Fig. 2.3, the pH titration curves of the histidine C-2(H) protons for both the Tyr-34_L and Tyr-33_H nitrated species are indistinguishable from those of the parent Fv fragments. Two of the histidines (His-102_H, pKa = 7.1, and His-97_L, pKa = 5.9; Wain-Hobson et al., 1977) are constituents of hypervariable loops which are in contact with the loops containing Tyr-33_H and Tyr-34_L respectively, and are therefore excellent markers to sense possible structural changes.

The changes in chemical shifts of the DNP-glycine protons on binding to the NO₂-Tyr-33_H Fv were determined by appropriate titrations (Dower et al., 1977). Since the hapten protons are in fast exchange the fully-bound chemical shifts may be obtained from Fig. 2.4 as described in section 2.2.6. These fully-bound shifts, together with those

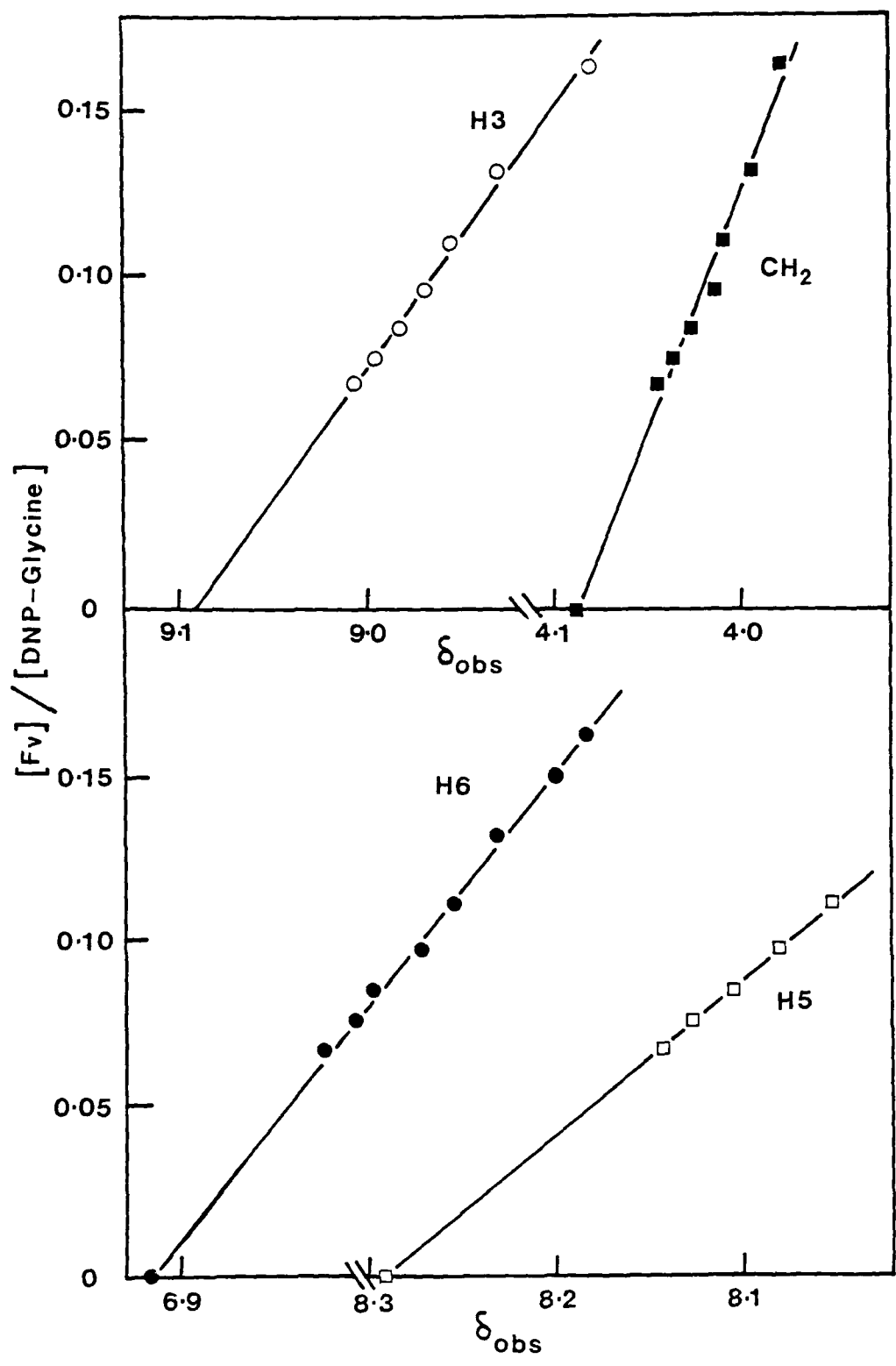


Figure 2.4 Change in chemical shift (at 470 MHz) of the protons of DNP-glycine on binding to NO_2 -Tyr-33_H Fv. The fully-bound chemical shift changes are given in Table 2.2. Conditions are $T = 30^\circ C$, protein concentration = 0.6 mM with 0.15 M NaCl, $pH^* = 8.1$, 1000 scans were accumulated per spectrum.

Table 2.1

Fully-bound chemical shift change on the protons of DNP-glycine on binding to Fv 315. All shift changes are upfield.

Proton	Change in chemical shift (ppm)		
	Fv ^a	NO ₂ -Tyr-34 _L ^b	NO ₂ -Tyr-33 _H
H(3)	1.21	1.3	1.3
H(5)	2.20	2.1	2.2
H(6)	1.77	1.5	1.4
CH ₂	1.00	0.8	0.7

^a Dower et al. (1977)

^b Gavish et al. (1979)

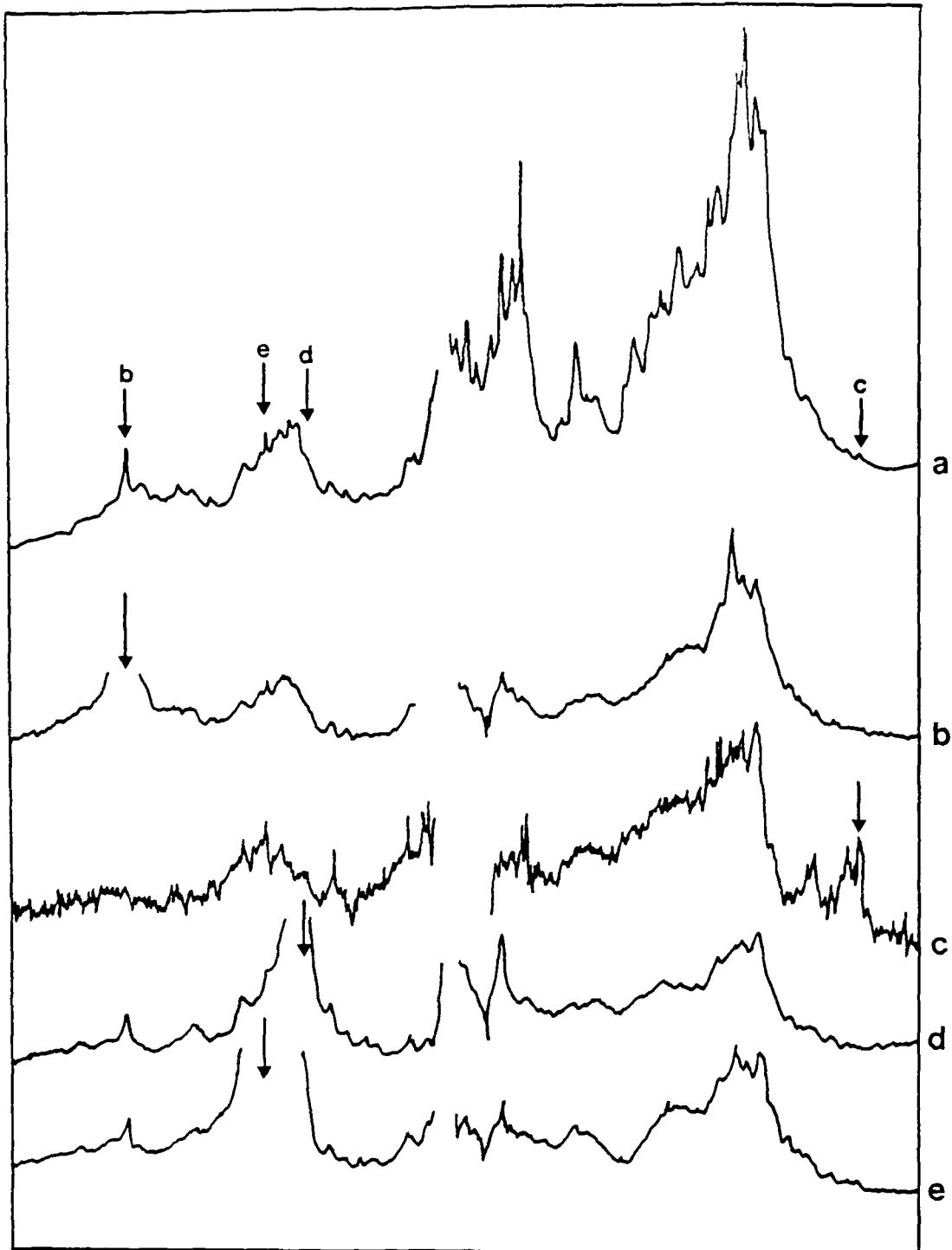


Figure 2.5 (a) 300 MHz ^1H NMR spectrum of Fv 315 + DNP-glycine. (b)-(e) Difference-NOE spectra produced upon irradiation of the spectrum at the indicated positions (arrowed). Protein concentration = 1 mM with 0.15 M NaCl, $\text{pH}^* = 7.2$, $T = 30^\circ\text{C}$. The experimental protocol is given in section 2.2.5.

obtained with NO₂-Tyr-34_L Fv (Gavish et al., 1979) and native Fv (Dower et al., 1977) are given in Table 2.1. The similarity of these values again indicates that the mode of binding of the hapten to each species is similar.

One of the criteria used by Dower et al. (1977) to place Tyr-34_L as a hapten contact residue was the reported observation that irradiation of a protein resonance at 6.6 ppm in a mixture of Fv and dinitrophenol resulted in a change in intensity of the hapten H(3) resonance. As it was assumed that the shift on this resonance was contributed largely by Tyr-34_L, the change in intensity was interpreted as a nuclear Overhauser effect (NOE) (Noggle & Schirmer, 1971) arising from proton(s) on Tyr-34_L. Since this is difficult to reconcile with the probable position of Tyr-34_L implied from the results of section 2.3.2, and because such an effect would provide a severe constraint on the positioning of this residue (or on whichever residue was responsible), the experiment was repeated using a DNP-glycine / Fv mixture. Spectra were accumulated in difference-NOE mode as described in section 2.2.5. The effect of irradiation at several positions in the spectrum (including the hapten H(3) position [b] and the reported specific NOE position at 6.6 ppm [d]) is shown in Fig. 2.5. It is evident that irradiation anywhere in the spectrum causes the same effect in the difference spectrum, and that in particular no specific NOE is observed between the H(3) and the protein. In control experiments, small molecular weight compounds displayed the expected NOEs (data not shown). Such observations on large molecular weight proteins have made previously, and are due to the longitudinal relaxation of individual protons in large molecules at high frequencies being largely influenced by cross-relaxation effects (Kalk & Berendson, 1976). It is concluded

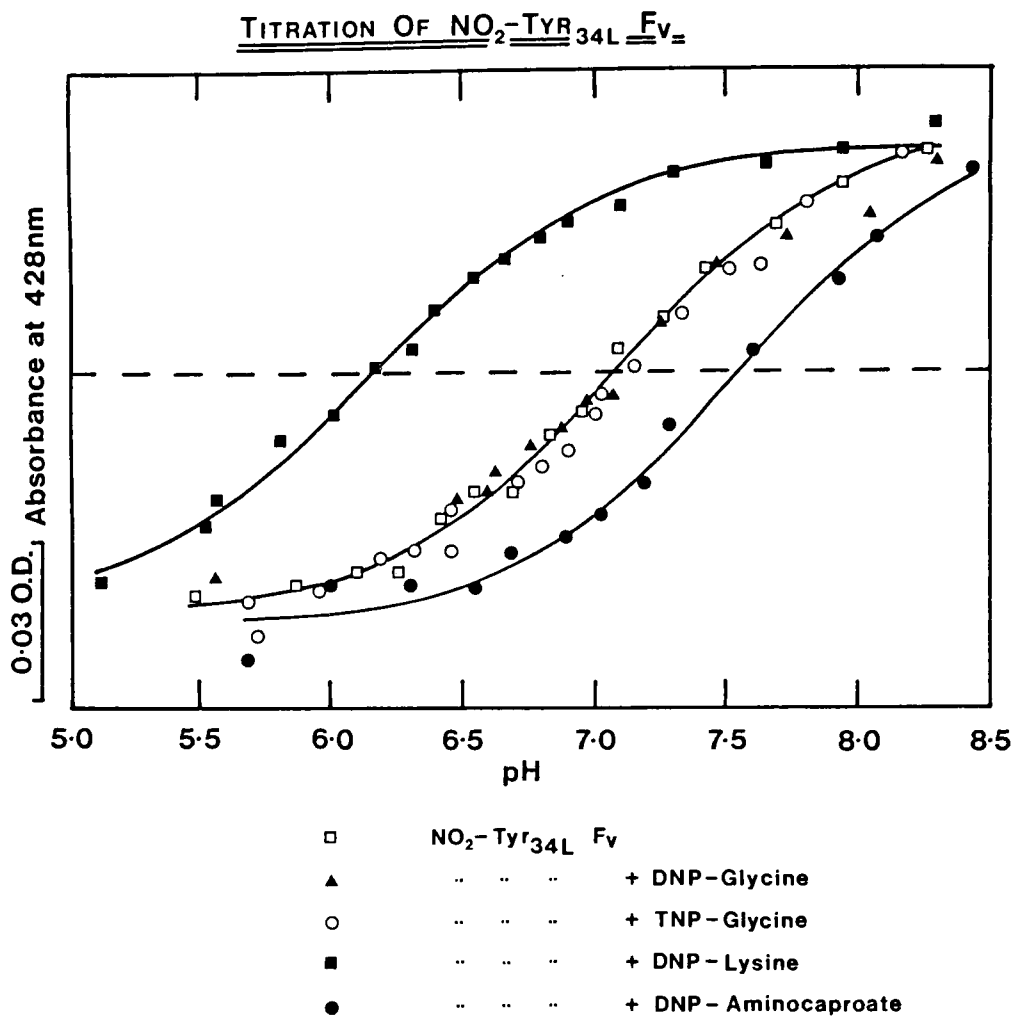


Figure 2.6 pH titrations of NO₂-Tyr-34_L in the NO₂-Tyr-34_L F_v fragment. Measurements were made at T = 293 K with solutions in 5 mM potassium phosphate / 0.15 M NaCl. Protein concentrations were 25 μM. Ligands were present in a slight molar excess. Since the hapten chromophores ($\lambda_{\text{max}} \sim 360$ nm) contribute to the absorbance at 428 nm, the different curves have been plotted using their ordinate scales offset such that the values of the absorbance at 428 nm at the pK of each titration curve is the same. This value is indicated by the dashed line (the value of the pK and the absorbance at this point were obtained by fitting the curve to a one-proton titration curve using the non-linear regression program PKA [appendix 1]). The titration parameters are summarized in Table 2.3.

that this effect is also encountered in the 25,000 dalton Fv fragment, and that therefore no easily interpretable steady-state NOEs may be obtained.

2.3.2 Effect of ligands on the ionization of nitrotyrosine-Fv

The results discussed in the previous section show that nitration of Tyr-33_H or Tyr-34_L affects neither ligand binding (including the binding constant [Gavish et al., 1979; Jackson, 1979]) nor the overall structure of the Fv fragment. Therefore these nitrotyrosine derivatives may be used to probe the interaction of ligands with their respective tyrosines in the native molecule. Furthermore, assuming that the DNP groups of different ligands bind in similar positions (Dower et al., 1977, 1978), the relative distances of the tyrosines from the DNP ring may be inferred by monitoring their interactions with a series of haptens having side chains of differing length.

The ionization of NO₂-Tyr-34_L, monitored at 428 nm follows a single-proton titration curve with a pKa of 7.1. This value is slightly higher than reported previously (Gavish et al., 1979), and similar to the value of 7.2 obtained with free 3-nitrotyrosine (Sokolovsky et al., 1967). The effects of various ligands on the ionization of this nitrotyrosine were determined using a protein concentration of 25 μM with a slight molar excess of ligand. The titration curves are shown in Fig. 2.6, and the titration parameters in terms of pKa and relative extinction coefficient, together with those obtained in a similar manner using NO₂-Tyr-33_H (Leatherbarrow et al., 1982) are given in Table 2.2.

Neither dinitroaniline nor trinitroaniline affect the ionization

Table 2.2

pKa values and relative extinction coefficients of NO₂-Tyr-33_H and NO₂-Tyr-34_L in the absence and presence of various nitrophenyl ligands^a

ligand	NO ₂ -Tyr-34 _L		NO ₂ -Tyr-33 _H ^b	
	relative		relative	
	pKa	E _{428 nm}	pKa	E _{428 nm}
none	7.1	1.0	7.4	1.0
DNP-NH ₂			7.5	0.9
TNP-NH ₂			7.7	1.15
DNP-NHCH ₂ COO ⁻	7.1	0.9	7.5	0.9
TNP-NHCH ₂ COO ⁻	7.1	1.0	8.1	1.5
TNP-NH(CH ₂) ₂ NH ₃ ⁺			7.7	2.15
DNP-NH(CH ₂) ₄ CH(NH ₃ ⁺)COO ⁻	6.2	0.9	7.0	1.25
DNP-NH(CH ₂) ₅ COO ⁻	7.5	1.0		

^a Measurements were made in 0.01 M potassium phosphate and 0.15 M NaCl; T = 293K. The protein concentrations were 25-50 μM.

^b from Leatherbarrow et al. (1982)

of $\text{NO}_2\text{-Tyr-33}_\text{H}$. Any observed effect may therefore be attributed to the side chain of the ligand. No such effect is seen with DNP-glycine, but the presence of TNP-glycine causes an increase in the pKa from 7.4 to 8.1 and a concomitant increase of the apparent extinction coefficient of the absorption band. An increase of the pKa would be expected from the proximity of the negatively charged carboxyl group of the ligand side chain. The difference between the effects of DNP-glycine and TNP-glycine may then reflect either a difference in the modes of binding of the DNP and TNP rings (Dower et al, 1978), or a steric effect of the 6-nitro group on the conformation of the side chain. That the perturbations are not solely due to simple charge effects is shown by the positively charged hapten TNP-aminoethylamine which, although demonstrably perturbing the nitrotyrosine, causes an increase in the pKa rather than the expected decrease.

Regardless of the mechanisms by which the perturbations are mediated, these results are consistent with the proximity of Tyr-33_H to the $\alpha\text{-CH}_2$ group of the ligand side chains, as originally proposed in the model of the combining site by Dwek et al. (1977). Since neither DNP-glycine nor TNP-glycine affect the pKa of $\text{NO}_2\text{-Tyr-34}_\text{L}$, it may be concluded that this residue is more distant from the $\alpha\text{-CH}_2$ group.

The addition of DNP-lysine reduces the pKa values of both nitrotyrosine derivatives. Presumably the flexibility of the long side chain can result in interactions involving both the nitrotyrosines. Although the pKa is reduced, it is difficult to separate the effects of the perturbations from the two charged end groups. However, the increase in pKa in the presence of DNP-aminocaproate, which is the same length as DNP-lysine, is as expected. It is pertinent to note that

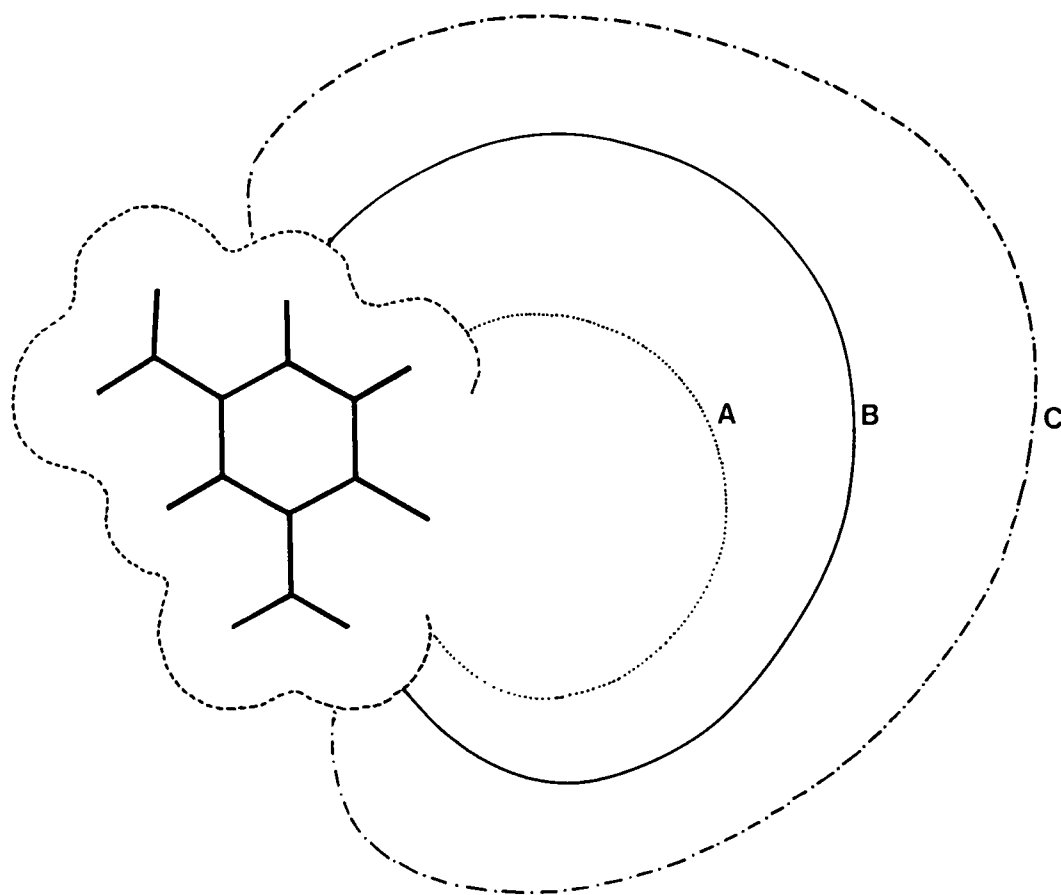
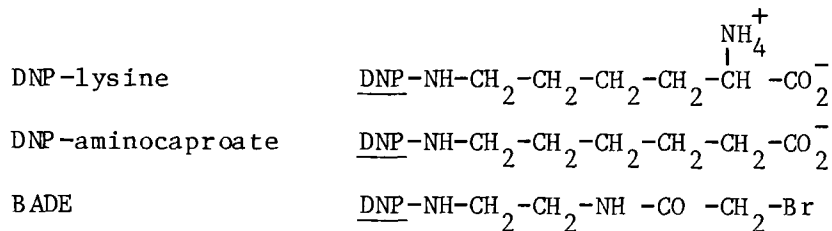


Figure 2.7 Illustration of the maximum area of interaction of:

- A the side chain of DNP-glycine/TNP-glycine
- B the reactive centre of the BADE affinity label
- C the side chain of DNP-lysine/DNP-aminocaproate

These distances were determined using standard Kendrew skeletal molecular models of the haptens together with space-filling components (scale 2 cm = 1 Å). Also shown is the DNP ring and its van der Waals surface.

Tyr-34_L can be affinity labelled with N-(bromoacetyl)N'-(dinitrophenyl)-ethylenediamine (BADE) (Haimovich et al., 1970, 1972) which is also approximately the same length as DNP-lysine, but less efficiently or not at all if the reactive bromoacetyl group is further from, or closer to, the DNP ring (Givol et al., 1971). This again implies that it is the charged terminal groups of the haptens that are perturbing the nitrotyrosine, as they occupy an analogous position to the reactive group of the affinity label:



2.3.3 The location of the side chain of Tyr-34_L

The above evidence, together with the fact that the DNP ring of covalently attached BADE-Tyr-34_L is still bound into the site (Givol et al., 1971), strongly suggests that the maximum distance that Tyr-34_L can be from the DNP ring is the length of the fully-extended side chains of the above haptens. The lack of any interaction with the side chain of DNP-glycine places a lower limit on the distance from the DNP ring. Using space-filling models of these haptens, the location of this tyrosine may be mapped relative to the DNP ring by tracing the possible positions of the charged side chains, and the affinity-labelling position of this tyrosine by BADE. As depicted in Fig. 2.7, this places severe limits on the location of the tyrosine ring. A similar analysis with the affinity label MNDB is not possible as its nitro-benzene group no longer occupies the combining site when it is covalently bound (as the modified protein is still able to bind hapten [Goetzl & Metzger,

1970]). However due to the relative shortness of this affinity label, and by consideration of Fig. 2.7, it would seem that the side chain of Tyr-34_L must be close to the 2-nitro group of the DNP ring (assuming MNDB binds in a similar manner to DNP, i.e. its nitro group is analogous to the 4-nitro group of DNP). The other constraint on the position of Tyr-34_L is that it can not be greater than 1.0 nm from Lys-52_H (part of the H2 hypervariable loop), as the bifunctional affinity reagent α -N-bromoacetyl- γ -N-DNP-L-diaminobutyric acid N'-bromoacetylhydrazide (DIBAB) will cross-link these residues (Givol et al., 1971), and its reactive groups are a maximum of 1.0 nm apart. The position of the phenolic ring of Tyr-34_L in the posulated site of Dwek et al. (1977) is shown in Fig. 1.15 (see also Fig. 3.4a). In this location it is difficult to envisage significant interaction with the side chain of DNP-lysine or DNP-aminocaproate, and it is not possible to have covalent interaction with BADE while the DNP ring still remains in the site. As has been discussed earlier, previous work on NO₂-Tyr-34_L (Gavish et al., 1979) has already found that ionization of the nitrophenolic group has no effect on hapten binding, showing that no hydrogen bond is formed between the phenolic -OH and the nitro groups of the haptens. It is concluded that the evidence presented in this chapter is consistent with the side chain of Tyr-34_L being located closer to the hapten side chains (i.e. further out of the site) than is suggested by the model of Dower et al. (1977).

2.3.4 Comparison with other immunoglobulin structures

The crystallographic structures of two Bence-Jones proteins, REI (Epp et al., 1974, 1975) and Mcg (Schiffer et al., 1973; Edmundson et al., 1974), which also have a tyrosine analogous to Tyr-34_L of M315

Table 2.3

Comparison of the distance of Tyr-34_L from the framework in REI and Mcg

Atom	Distance to Tyr-34 _L (Å)			
	REI		Mcg	
	C ^α	O ^η	C ^α	O ^η
C ^α Trp-37 _L	10.3	16.3	10.0	16.5
C ^α Cys-90 _L	10.3	16.7	10.5	16.7

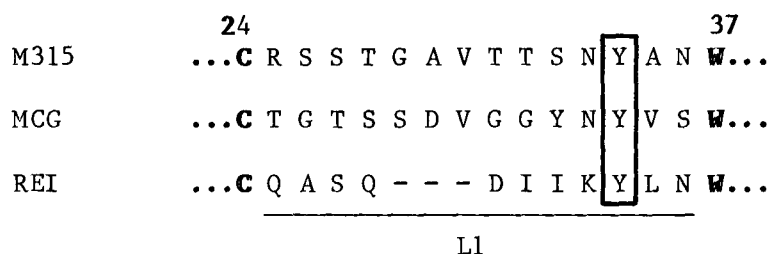
Table 2.4

Comparison of the distance of Tyr-34_L from the first hypervariable residue of loops L1 and L3

Atom	Distance to Tyr-34 _L (Å)					
	REI		Mcg		M315 ^a	
	C ^α	O ^η	C ^α	O ^η	C ^α	O ^η
C ^α Asn-36 _L	6.9	12.8	6.3	12.2	6.0	10.6
C ^α Ala-91 _L	8.3	14.4	8.0	14.0	6.9	12.1

^a from the proposed site of Dwek et al (1977)

are known. The amino acid sequences of the L1 loops from these proteins are given below (M315 numbering) (Kabat et al., 1979)



Since the length of the L1 hypervariable loops of MCG and M315 are the same, and the sequence around the C-terminal end of the loop is similar, the positions of Tyr-34_L may also be expected to be similar. The structure in REI may give a clue as to whether any variation is found, since although it contains an analogous tyrosine, the loop is shorter by three residues, and the amino acid sequence around the C-terminal end is less like that of MCG or M315. A comparison of the position of this tyrosine in these proteins with that proposed for M315 (Dwek et al., 1977) may then provide an indication as to the accuracy of its predicted location in protein 315.

The distances of these tyrosines from the alpha-carbon atom of the invariant framework residues Trp-37_L and Cys-90_L (from the neighbouring hypervariable loop) are given in Table 2.3. These distances were calculated from the crystallographic coordinates. For REI, the average value of the distances in both halves of the dimer were used (the individual values are very similar). For MCG, since crystal contacts distort the position of the Tyr-34_L side chain in one of the loops only the undistorted structure was used. The distances in both REI and MCG are found to be almost identical, showing that their structure up to Tyr-34_L is probably the same. This allows both these structures to be used as a reference with which to compare the L1 loop of M315, and

implies that the position of this tyrosine in M315 should also be the same. Unfortunately no framework coordinates are available for the structure of Dwek et al. (1977), and so the distance from the hypervariable residue adjacent to the framework was calculated. These values are given in Table 2.4. REI and Mcg are again found to give almost identical values, however the predicted structure of M315 differs from these structures, and significantly the η -oxygen atom is $\sim 2 \text{ \AA}$ too close to the framework. It should be noted that it is the ionization of this hydroxyl group in the nitrotyrosyl derivatives which is sensitive to the charged side chains of the haptens.

It is concluded that the position of the side chain of Tyr-34_L proposed by Dwek et al. (1977) is incorrect. Relocation by $\sim 2 \text{ \AA}$ towards the front of the site to a position equivalent to that in REI and Mcg would make the location of the side chain consistent with the nitration and affinity labelling results discussed above. This still leaves the side chain of Tyr-34_L close to, and approximately in the plane of the DNP ring - a position which is also indicated by the downfield shift of 0.34 ppm on the resonance tentatively assigned to this tyrosine on binding hapten (section 2.3.1).

2.4 SUMMARY

In conclusion, the results presented in this chapter suggest that Tyr-33_H occupies a position which is compatible with that proposed by Dwek et al. (1977), whereas Tyr-34_L is more peripheral to the site than suggested by that model. This latter conclusion is consistent with other labelling studies (Goetzl & Metzger 1970a,b; Haimovich et al., 1970, 1972; Givol et al., 1971) and previous work on NO₂-Tyr-34_L (Gavish et al., 1979). Placing the side chain of Tyr-34_L in a position which is similar to that of the analogous tyrosine in REI and Mcg (i.e. ~ 2 Å further out of the site) is compatible with all the modification data. This would still leave Tyr-34_L as a hapten contact residue, but not in such intimate association as proposed by Dwek et al. (1977) and not hydrogen-bonded to the 2-nitro-group of the hapten. The effect that this has on any postulated model of the combining site of protein 315 is discussed in the following chapter.

CHAPTER 3

Model-building of Antibody Combining Sites. A Refined Model of the Site of Protein 315

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3.1 INTRODUCTION

3.1.1 Prediction of combining-site structures

As was discussed in chapter 1, it is apparent that the tertiary structures of the non-hypervariable regions of immunoglobulin V-domains are very similar. In view of this structural invariance, the variable region may be regarded as a rigid framework to which the hypervariable loops are attached. The tertiary structure of these loops defines the combining site. If these structures could be predicted from their amino acid sequence, then it might be possible to obtain information about the combining site of immunoglobulins of known sequence, but for which crystallographic data is not available. Such structural prediction is simplified to some extent by the small size of the loops (at most 17 residues), and by the rigidly defined positions at which they begin and end.

Prediction of hypervariable loop conformations has been performed by two methods:

1. Model-building to maximize structural homology with similar hypervariable regions of known tertiary structure. This has been performed both manually (Padlan et al., 1976; Davies & Padlan, 1977), and using computer assistance (Feldmann et al., 1981).
2. Empirical prediction of the $\{\varphi, \psi\}$ angles of amino acid triplets within the loops (Stanford & Wu, 1981; Coutre et al., 1981) to obtain a possible conformation for the alpha-carbon backbone.

The first method as applied by Padlan et al. and Davies & Padlan assumes that hypervariable loops of similar length will have similar tertiary structure. While this cannot be absolutely correct, just how much

inaccuracy this causes is not possible to predict. The second method utilized an empirical procedure for prediction of protein secondary / tertiary structure. Such a simple procedure is at best only useful for identifying possible regions of secondary structure in a whole protein. It is not likely to be useful for application to hypervariable loops, where the constraints applied by their short length and fixed termini mean that a regular secondary structure is unlikely. In view of this it is probable that the site of protein 315 as described by Padlan et al. (1976) from model-building is more accurate than the quite dissimilar structure given by Stanford & Wu (1981) from prediction of $\{\varphi, \psi\}$ angles. These two predicted structures are compared in greater detail in section 3.3.3.

As described in section 1.10, the model by Padlan et al. (1976) of the combining site of protein 315 has been used as a starting point for structural refinement by Dwek and co-workers (Dower et al., 1977; Dwek et al., 1977) of the positions of the constituent amino acid side-chains. Although utilizing numerous experimental data, the main criteria used for defining this refined site were the changes in chemical shift experienced by haptens on binding to the protein. Geometries consistent with the bound chemical shifts were obtained by calculation of theoretical shift changes caused by the proximity of amino acid side chains to the bound hapten. The justification and probable accuracy of such an approach are examined below.

3.1.2 Causes of chemical shift changes on hapten binding

Before it is possible to interpret the changes in chemical shift of a hapten when bound to a protein, it is necessary to consider the

various factors which may be responsible for the shifts, and to assess the potential magnitude of these contributions (for a more detailed review see Perkins, 1982). These factors are separable into through-space effects, and changes in intramolecular electron distribution. The former is due to local changes in the magnetic field, and acts on the ^1H nucleus directly. In proteins such changes arise mainly from conjugated systems, namely amides, carbonyls, guanidino groups (arginine side chains) and aromatic groups (the side chains of histidine, phenylalanine, tryptophan and tyrosine). With aromatic groups such effects are termed "ring-current" shifts. The calculation of these ring-current shifts may be performed by various treatments, which are described in appendix 2.

Changes in intramolecular electron distribution can also alter the shielding of a proton. These changes are either caused by local electric field effects, such as from charged groups on the protein, or by an alteration in the reaction field (Buckingham, 1960) of the ligand on binding. The reaction field arises from a dipole induced polarization in the solvent surrounding a solute which has a dipole moment. This would be expected to occur in aqueous solution, but differ in the more apolar environment of the combining site i.e. where the local magnetic susceptibility is different. Such changes may be demonstrated by changing the solvent. For DNP-aspartate it has been shown that whereas the chemical shift of H(3) and H(5) are largely independent of solvent ($|\Delta\delta| < 0.1$ ppm), the H(6) resonance moves upfield by as much as 0.3 ppm with decreasing solvent polarity over the series $^2\text{H}_2\text{O}$ (pH* 3.5), acetic acid, methanol, acetone (Wain-Hobson, 1977).

Of the various factors described above the most significant effects are those produced by the presence of an aromatic ring. In comparison the magnitude and range of the chemical shift change produced by the other above effects is small (Perkins, 1982). Therefore apart from certain special cases (such as amino acid alpha-carbon atom protons which are very close to the peptide carbonyl groups, and NH protons where hydrogen bonding markedly affects the chemical shift) it is reasonable to assume that the secondary chemical shift experienced by a protein proton is due to ring-currents, with the expected contribution from other factors being of the order of $\leq \pm 0.2$ ppm.

3.1.3 Reliability of ring-current calculations

Although it is clear that ring-currents are the major contributory factor in secondary shift changes, the magnitude of the shifts produced needs to be calculated with some degree of accuracy for this to be useful in structural refinement. It is therefore most opportune that several recent comparisons have been made between the observed secondary shifts in proteins and those calculated from the crystallographic structure using ring-current equations (Perkins & Wüthrich, 1979; Perkins & Dwek, 1980; Perkins & Wüthrich, 1980). The results from these studies are summarized as follows:

1. There is good correlation between observed secondary shifts and those calculated using ring-current theories. This substantiates that ring-currents are the main cause of such effects.
2. While the treatments of Johnson-Bovey and Haigh-Mallion (see appendix 2), each allow the data to be fitted equally well, the Pople point dipole approximation leads to a poorer estimation of the experimental chemical shifts (Perkins, 1982).

3. Although the slope of the plot of observed versus calculated values is unity for shifts from tyrosyl and phenylalanyl rings, this is not the case for shifts from tryptophanyl rings. In the latter case the calculated shift consistently underestimates the observed values. This may be resolved by increasing the empirical intensity factors (see appendix 2) for the five and six membered rings of the tryptophan indole system. The optimal values for these intensity factors to give correspondence with the observed shifts are 1.2 and 1.7 for the six and five membered rings respectively (the previous values were 1.04 and 0.56 [Guissner-Prettre & Pullman, 1969]). This effectively treats the tryptophan system as two fused benzene rings.
4. While the overall correlation between observed and theoretical shifts is good, individual points may differ from the calculated value. The largest observed deviation so far (ignoring alpha-carbon atom and NH protons) is less than 0.3 ppm (Perkins, 1982). The root mean square deviation is 0.1-0.2 ppm.

The discrepancy between the observed and theoretical shifts is due to four factors. Firstly the resolution of the X-ray crystallographic structure may be inadequate - the ring-current shift on a proton close to an aromatic ring is very sensitive to position. Secondly, the crystallographic structure represents a time-averaged conformation. The non-linear dependence of chemical shift on distance means that calculations based on this average conformation may lead to incorrect calculated chemical shifts due to the influence of the dynamic motion of the protein. Thirdly, other factors besides ring-currents may be involved. As discussed in the previous section, the magnitude of the effects produced by such factors is comparable with these observed deviations. Finally, the ring-current theories themselves may be

inadequate, particularly when non-benzenoid systems are involved. This is exemplified by the underestimation of shifts from tryptophan rings, but not from the simpler tyrosyl and phenylalanyl rings.

The recalibration of intensity factors as described above offers an empirical solution, and allows experimental results to be predicted with greater accuracy. A possible physical basis for the lack of agreement between experiment and ring-current theory for tryptophan is that for this ring system there is significant diamagnetic anisotropy effects due to the presence of the nitrogen atom in the ring (Guessner-Prettre & Pullman, 1981). However, whatever the cause, the treatment of Perkins & Dwek (1980) offers the best compromise between calculated and observed results, and therefore was the one used to calculate all subsequent geometries described in this chapter.

3.1.4 Applicability of ring-currents to predict the site of M315

The considerations of the preceding sections show that in general the secondary shift on a proton in the environment of protein side chains may be explained in by ring-current effects, with an expected accuracy of $\sim \pm 0.2$ ppm. This means that for the small secondary shifts often observed in proteins ($|\Delta\delta| < 0.3$ ppm), ring-current theory is little help in providing structural information (Cassels et al., 1978; Dobson et al., 1978). However for protein 315 the changes in chemical shift of the hapten protons are large ($\Delta\delta = 1-2.5$ ppm), and such an inaccuracy is insignificant in determining the location of a proton relative to surrounding aromatic rings (a variation of 0.2 ppm to a shift of 1 ppm produced by a benzenoid ring is accounted for by a movement of only ~ 0.02 nm). Direct evidence for the involvement of

aromatic rings in the site of protein 315 is provided by the mainly aromatic perturbations on hapten binding (Dower et al., 1977).

The limitations of this method are twofold. Firstly the calculation of the ring-current shifts is semi-empirical, the approximations involved being worsened by the necessarily close approach of hapten protons to the aromatics in the site. Secondly no unique site could be predicted from ring-current considerations alone. A model of the site is required as a first approximation. The possible models available for the site of protein 315 are considered in section 3.3.3.

3.2 MATERIALS AND METHODS

3.2.1 Model-building

Models of combining site structures were built using standard Kendrew skeletal components (Cambridge Repetition Engineers) on a scale $2 \text{ cm} = 0.1 \text{ nm}$. Where necessary, scale van der Waals surfaces were added to prevent unfavourable inter-atomic distances. Co-ordinates were measured using a plumb-line, and then regularized to standard bond lengths and angles by the computer program MODELFIT written by N. Isaacs, and kindly made available by Dr K. Wilson, Department of Molecular Biophysics, Oxford. Co-ordinates of the final predicted combining site of protein 315 may be found in microfiche form attached to the inside back cover of this thesis.

3.2.2 Calculation of ring-current shifts

Ring-current shifts were calculated using the Johnson-Bovey equation in one of three ways: 1. Manually, using scale contour maps of ring-current shift together with scale models of haptens (Dower et al., 1977). This allows rapid screening of various geometries before accurate calculation. 2. By use of tables of ring-current shifts (Johnson & Bovey, 1958; Perkins et al., 1977). 3. By computer calculation in which the chemical shift changes were determined by use of the FORTRAN 77 program RINGCALC (appendix 1) from co-ordinates of aromatic residues and hapten protons.

3.2.3 Model of the L-chain dimer of M315

The rotation and translation matrices relating the monomers of the dimeric light chain REI were obtained by superposition of the alpha-carbon co-ordinates of the two monomers. This was achieved by use of the FORTRAN IV program SUPERB written by Dr J. Thornton, and kindly made available by Dr K. Wilson, Department of Molecular Biophysics, Oxford. These matrices were then used to produce a hypothetical second monomer from the co-ordinates of the proposed 315 model (these co-ordinates had first been transformed to those of REI by superposition of the homologous L2 and L3 hypervariable loops by use of the SUPERB program, because the co-ordinate systems of the proteins are different).

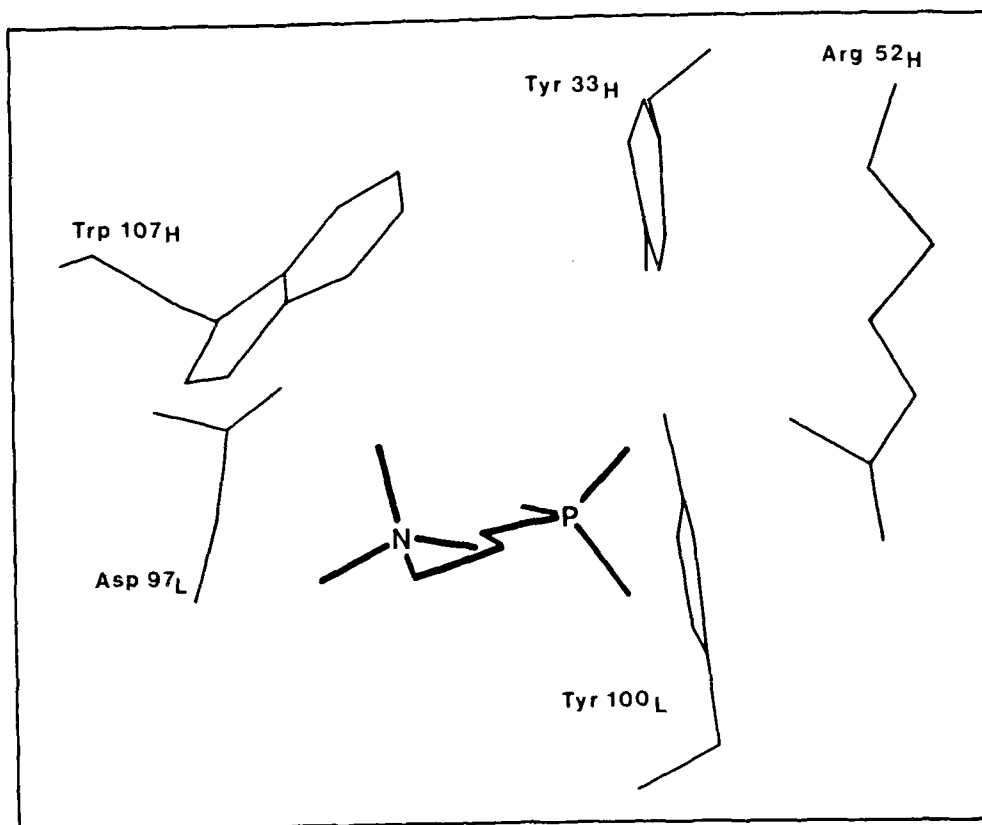


Figure 3.1 The combining site of M603 containing the hapten phosphocholine (diagram kindly supplied by D. R. Davies).

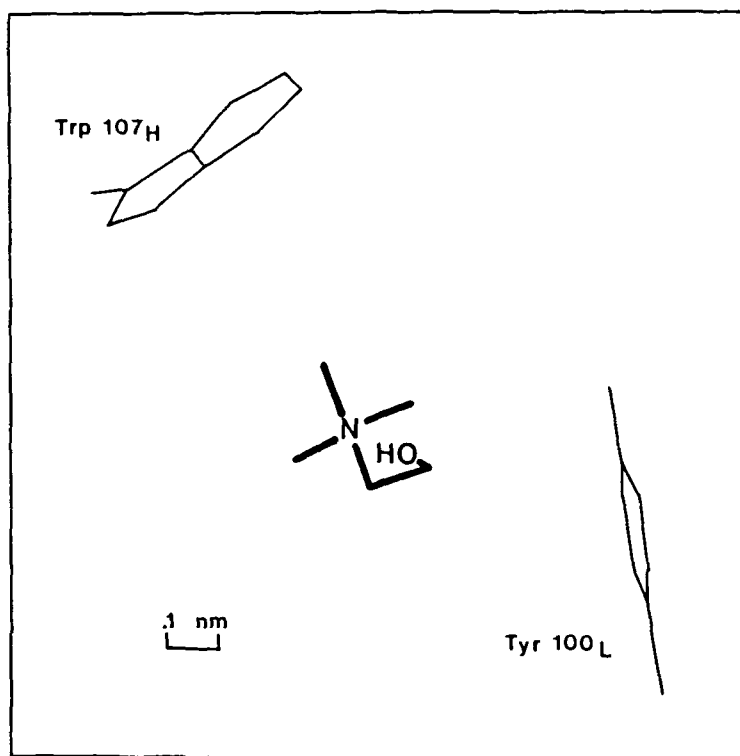


Figure 3.2 Position of aromatic residues around a choline hapten which are consistent with the experimental shift changes (Tables 3.1 and 3.2).

3.3 RESULTS AND DISCUSSION

3.3.1 Comparison of calculated and observed shifts for M603

It is clear that an important test of the validity of the ring-current approach to predict antibody combining sites would be a comparison of calculated and experimental chemical shift changes on a hapten, when binding to an antibody fragment where the structure of the antibody-hapten complex is known. The structure of the Fab' fragment from the phosphocholine-binding myeloma protein M603 (Padlan et al., 1973; Segal et al., 1974) is currently known to a resolution of 0.27 nm (D. R. Davies, personal communication) and the location of phosphocholine has been obtained from a 0.31 nm difference Fourier map (E. A. Padlan & D. R. Davies, personal communication). The hapten contact residues in the site are shown in Fig. 3.1. High-resolution NMR studies on hapten binding to the Fab' fragment of M603 have been performed (Gettins et al., 1982), and the fully-bound chemical shift changes for choline are given in Table 3.1 (phosphocholine binds very tightly to M603 [$K_d = 5 \mu\text{M}$, Gettins et al., 1977] and hapten and protein proton resonances are in slow exchange at 270 MHz. Choline has a lower affinity [$K_d = 1.2 \text{ mM}$, Goetze & Richards, 1977], and the proton resonances are in fast exchange, allowing the fully-bound shift changes to be obtained.). It is therefore possible to assess whether the NMR results are consistent with the known structure of the complex.

Examination of the difference spectrum produced on binding of phosphocholine to the Fab' fragment reveals no large structural perturbations (Gettins et al., 1977). This is substantiated by the crystallographic studies which show that no significant conformational

Table 3.1

Changes in chemical shift of the three proton resonances of choline chloride on binding to M603 Fab'. A plus sign represents an upfield shift. (Data from Gettins et al. 1982)

	⁺ NMe ₃	α-CH ₂	β-CH ₂
Shift ratio	1.00	0.73	0.38
Fully-bound shift change	+0.88	+0.64*	+0.34*

* These values were determined by multiplying the value of +0.88 ppm for the methyl resonance by the observed shift ratio.

Table 3.2

Ring current shifts (ppm) calculated from Trp-107_H and Tyr-100_L on choline as shown in Fig. 3.2. The values are calculated using the method of Johnson & Bovey (1958), with scaling factors for tryptophan as in Perkins & Dwek (1980).

	⁺ NMe ₃	α-CH ₂	β-CH ₂
Tyrosine-100 _L	+0.13*	+0.27	+0.16
Tryptophan-107 _H	+0.63*	+0.27	+0.25
Total	+0.76*	+0.54	+0.41

* Average of several values allowing for rotation of the ⁺NMe₃ group.

changes occur on binding. In the difference spectrum, 6-10 aromatic and 11-14 aliphatic protons are visibly perturbed. This is in reasonable agreement with the composition of the constituent groups involved directly in hapten contact (Fig. 3.1). The fully-bound chemical shifts of the choline protons (Table 3.1) are all large and upfield. Given that the crystal structure places the aromatic side chains of Trp-107_H, Tyr-33_H and Tyr-100_L in contact with the hapten, it is reasonable to assume that a large proportion of the observed shift changes are due to ring-current effects. Assuming that the N⁺Me₃ group in choline occupies an analogous position in the site to this same grouping in phosphocholine, a model of it was built surrounded by these aromatic side chains, with relative orientations as in Fig. 3.1. Tyr-33_H is found to be too distant (> 0.6 nm) from any of the hapten protons to significantly contribute to the shift changes. Therefore the experimental data were explained on the basis of ring currents from Trp-107_H and Tyr-100_L alone. The positions of the side chains of these residues which produce the best agreement between the calculated and experimental shift changes are shown in Fig. 3.2. The calculated shifts for this arrangement are given in Table 3.2.

It is apparent from these results that there is good agreement between the predicted and observed values ($|\Delta\delta_{\text{exp.}} - \Delta\delta_{\text{calc.}}| \leq 0.12$ ppm). The discrepancy is quite small, and within the range of the expected accuracy of such calculations (discussed in section 3.1). Calculations based on ring current considerations alone (using the treatment of Perkins & Dwek, 1980) are therefore shown, at least in this system, to provide a reasonable explanation of the orientations of the surrounding aromatic residues. The interactions of haptens with the charged residues of this site have been monitored using ³¹P-NMR on

phosphocholine and its phosphonium analogue (Gettins et al., 1977, 1982). These results are also explained by the crystal structure, in which there is a negatively charged residue [Asp-97_L] near to the $-N^+-Me_3$ group, and a positively charged residue [Arg-95_L] next to the $-OPO_3^-$ group. It is concluded that interpretable information on the orientation of residues in an antibody combining site can be obtained from NMR studies. However, this is only true if the model of the site used as a starting point for the calculations is a good approximation to the actual site. This latter point is obviously most relevant to any structural predictions on the combining site of protein 315. It is important to note that if the treatment of ring-current shifts by Perkins et al. (1977) had been used, it would not have been possible to obtain a satisfactory agreement between the observed and calculated chemical shift changes for M603.

3.3.2 Ring-current contributions by tryptophan in the site of M315

It is well established that a major contribution to the specificity of protein 315 is the involvement of a tryptophan side chain in a stacking interaction with the DNP ring (see section 1.10.2). It is therefore to be expected that a large proportion of the shifts experienced by the DNP ring protons on binding to protein 315 are due to ring-current shifts from this tryptophan. In the light of the re-evaluation of the intensity of these effects from tryptophan residues (section 3.1.3) it is necessary to examine what difference (if any) this makes to structural prediction of the combining site using ring-current calculations.

It is to be expected that the DNP ring will lie parallel to and

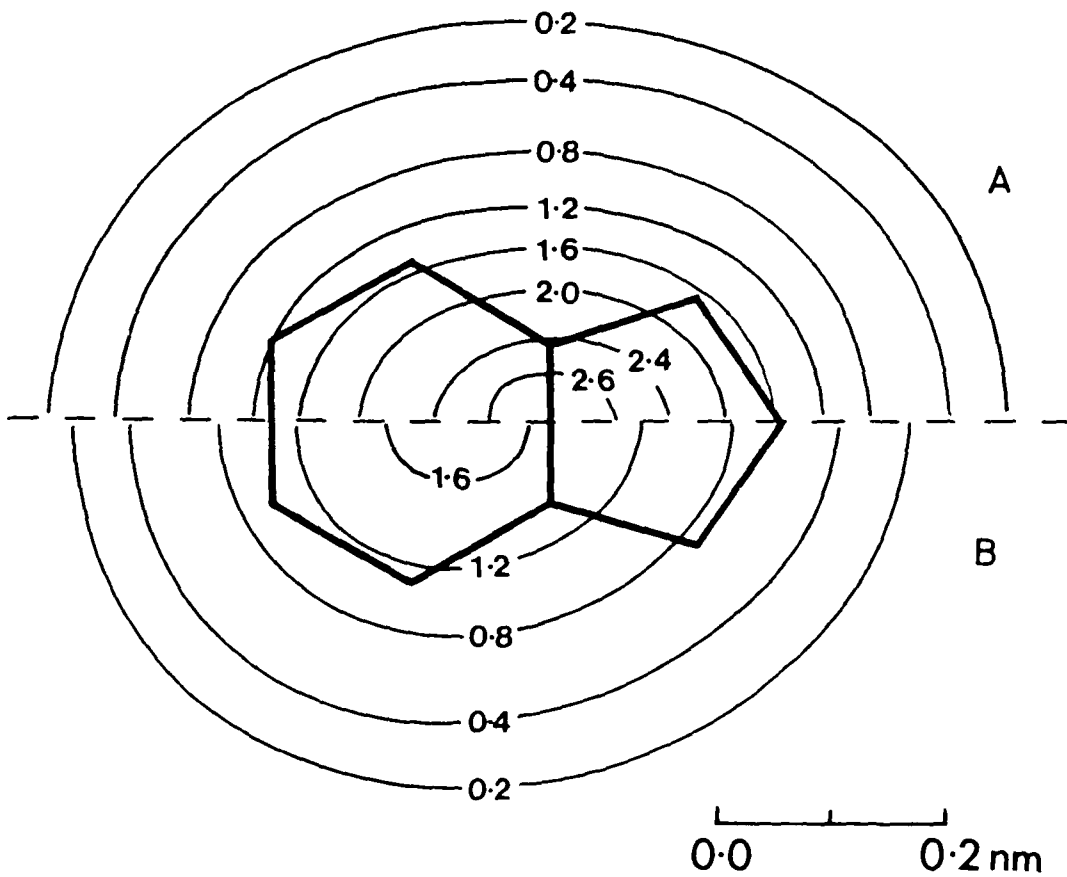


Figure 3.3 Comparison of the calculated ring-current contours (in ppm) from a tryptophan residue at 0.33 nm above the plane of the ring using the treatments in (A) Perkins & Dwek (1980) and in (B) Perkins et al. (1977).

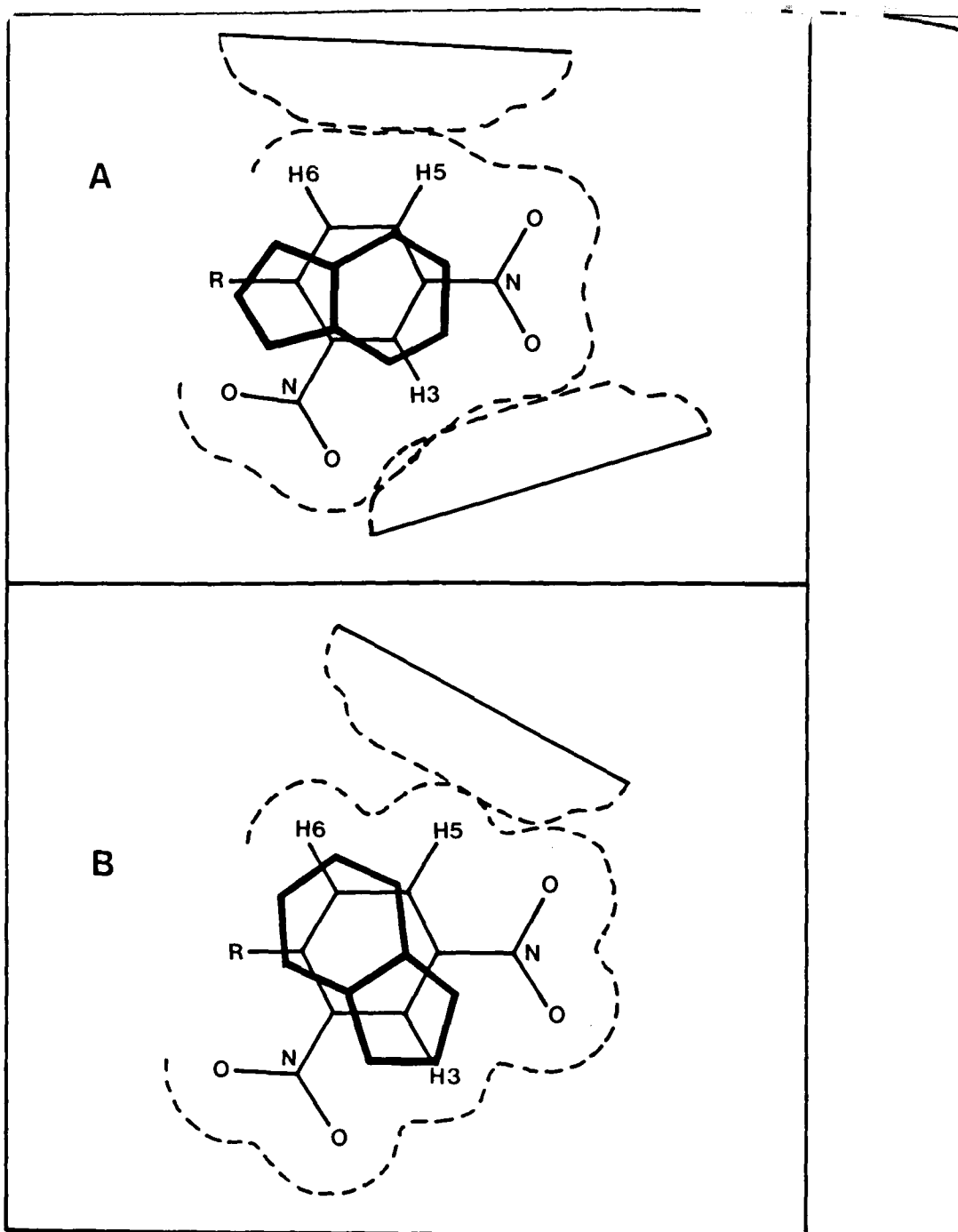


Figure 3.4 The minimum requirement of neighbouring aromatic residues to account for the experimental chemical shift changes on the DNP hapten ring protons when binding to protein 315. The DNP ring is assumed to lie parallel to and 0.33 nm from the tryptophan ring, and the surrounding benzenoid rings are perpendicular to the DNP ring. The dotted lines represent van der Waals surfaces. (a) Obtained by Dower *et al.* (1977) using the ring-current treatment of Perkins *et al.* (1977). (b) Obtained using the ring-current treatment of Perkins & Dwek (1980).

0.33 nm from the tryptophan ring, i.e. at the distance of closest approach between two aromatic rings. This is the separation found in crystallographic studies on similar model compounds (Hanson, 1964; Gartland et al., 1974). A comparison of the ring-current shifts at this distance as calculated from the treatments of Perkins et al. (1977) and Perkins & Dwek (1980) is shown in Fig. 3.3. Both treatments by the nature of the Johnson-Bovey equation predict contours which are symmetrical around the long axis of the ring. The later treatment however, gives a field which is also symmetrical about the mid-line of the ring system. In addition, the magnitude of the predicted shift is greater, particularly from the five-membered portion of the ring.

The consequence of this for predicting the arrangement of aromatic residues around the DNP ring is as follows. The treatment of Perkins et al. (1977) as used by Dower et al. (1977) and Dwek et al. (1977) necessitated a minimum of a tryptophan ring and two additional aromatic rings to account for the shifts on the hapten ring protons. In contrast, by use of the treatment of Perkins & Dwek (1980), the minimum requirement is now found to be a tryptophan together with a single additional aromatic ring. This additional ring must be positioned close to the H(5) and H(6) protons in order to produce the observed shifts on these protons, the shift on the H(3) proton is contributed solely from the tryptophan. A geometry which satisfies these requirements is shown in Fig. 3.4, where it is compared with that described as the minimal requirement by Dower et al. (1977). It is clear that different predicted sites may be obtained using the different treatments.

This basic arrangement (Fig. 3.4b) can be used to account for the shift changes observed on binding a variety of different ligands.

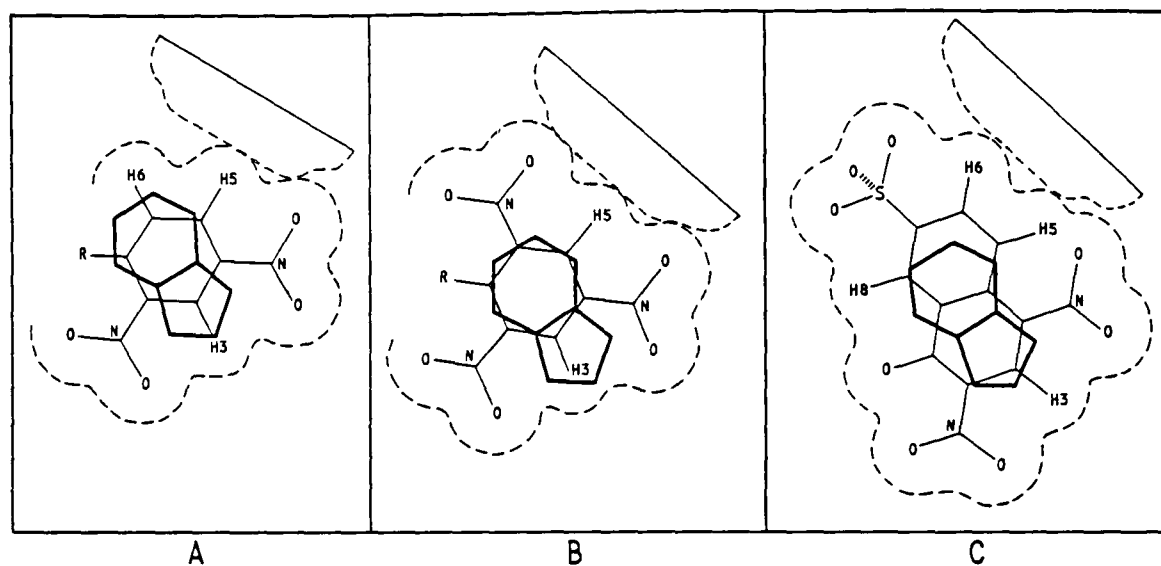


Figure 3.5 Positioning of aromatic rings to account for the experimental shifts on DNNS, DNP-aspartate, and TNP-aspartate. The haptene ring is positioned 0.33 nm from and parallel to the tryptophan ring, and perpendicular to the adjacent aromatic ring. Van der Waals surfaces (dashed lines) of the haptens and adjacent aromatic rings are indicated.

- (A) DNP-aspartate
- (B) TNP-aspartate
- (C) DNNS

Fig. 3.5 shows arrangements which satisfy the experimental results for DNP-aspartate, TNP-glycine and DNNS, which are representative of the wide range of compounds bound by protein 315. The experimental and theoretical shifts are given in Table 3.3. That this arrangement satisfies such diverse compounds with minimal rearrangements is good evidence for such a structure being found in the protein. This can be built into a model of the combining site (providing of course that the amino acid sequence is compatible with such an arrangement of aromatic side chains), but first a suitable prediction for the structure of the site must be available.

3.3.3 The choice of a starting model for refinement of M315

As has been emphasized previously, refinement of a proposed combining site model to make it compatible with physical studies is extremely difficult unless the original proposed site is a reasonably close approximation of the actual site. With protein 315 two predicted sites are available - Padlan et al. (1976) and Stanford & Wu (1981). Previous structural refinement was based on the site of Padlan et al. (Dower et al., 1977), this being the only one available at the time. In order to ascertain whether the site of Stanford & Wu might be a more appropriate starting point, a model of the peptide backbone was built (no side chain positions had been considered in this prediction), side chains were added, and it was attempted to use the resultant arrangement to account for the experimental data. Trp-93_L was taken to be the tryptophanyl residue involved in a stacking interaction with the DNP ring. It was found to be very difficult to find a suitable arrangement of surrounding aromatic residues which explained the shifts on the hapten ring protons, and especially difficult to account for the 1 ppm

Table 3.3

Comparison of calculated^a and observed chemical shift changes of the resonances of various hapten ring protons on binding to the Fv fragment of M315

hapten	proton	exptl	shift change		
			calcd		
			Trp	Phe	total
DNNS ^b	H(3)	0.84	0.83	0.0	0.83
	H(5)	1.07	0.40	0.6	1.00
	H(6)	1.72	0.17	1.7	1.87
	H(8)	0.65	0.55	0.0	0.55
TNP-aspartate ^c	H(3) + H(5)	2.38	1.3	0.9	2.2
DNP-aspartate ^d	H(3)	1.68	1.6	0.0	1.6
	H(5)	2.3	0.6	1.8	2.4
	H(6)	1.3	0.9	0.5	1.4

^a Using the positions of aromatic rings in Fig. 3.5

^b From Wain-Hobson (1977)

^c From Dower et al. (1978)

^d From Dower et al. (1977)

shift on the $\alpha\text{-CH}_2$ group of DNP-glycine. The nitration results of chapter 2 show that the side chain of Tyr-33_H is close enough to this grouping to produce the additional required shift (~ 0.6 ppm is required as Trp-93_L cannot contribute more than ~ 0.4 ppm), as was predicted from the site of Padlan *et al.* (1976) (Dower *et al.*, 1977). In the model of Stanford & Wu this residue is ~ 1.3 nm from the DNP side chain. Repositioning it correctly would require quite extensive re-building. In view of this, and because of the difficulty in explaining the experimental data, it would seem that the original prediction of Padlan *et al.* (1976) is a better approximation of the actual site. Since the alterations made to this site in order to produce a refined site of protein 315 (Dower *et al.*, 1977) were minimal, and were made to account for the NMR data better (e.g. the lack of aliphatic perturbations on hapten binding meant that Leu-103_H was moved out of the site), it was the site of Dower *et al.* that was taken as a starting point for further modification, using the results on the position of Tyr-34_L (chapter 2), and the increased ring-current shift from tryptophan (section 3.3.2).

3.3.4 A refined model of the combining site of M315

As a consequence of the chosen starting model of the site, the extra aromatic ring depicted in Fig. 3.4b is essentially limited to Phe-34_H. Other surrounding aromatics (Phe-98_L, Tyr-33_H, Trp-35_H, Phe-50_H, Tyr-104_H, Phe-105_H) are too distant to be involved without quite major rearrangement of the site. Tyr-34_L is also a candidate, but chemical modification data (affinity labelling by both BADE and MNDB together with the results in chapter 2) suggest its location near to the 2-nitro group (see section 2.3.3), i.e. not close to H(5) and H(6). The tryptophanyl side chain must again be from Trp-93_L. These residues may

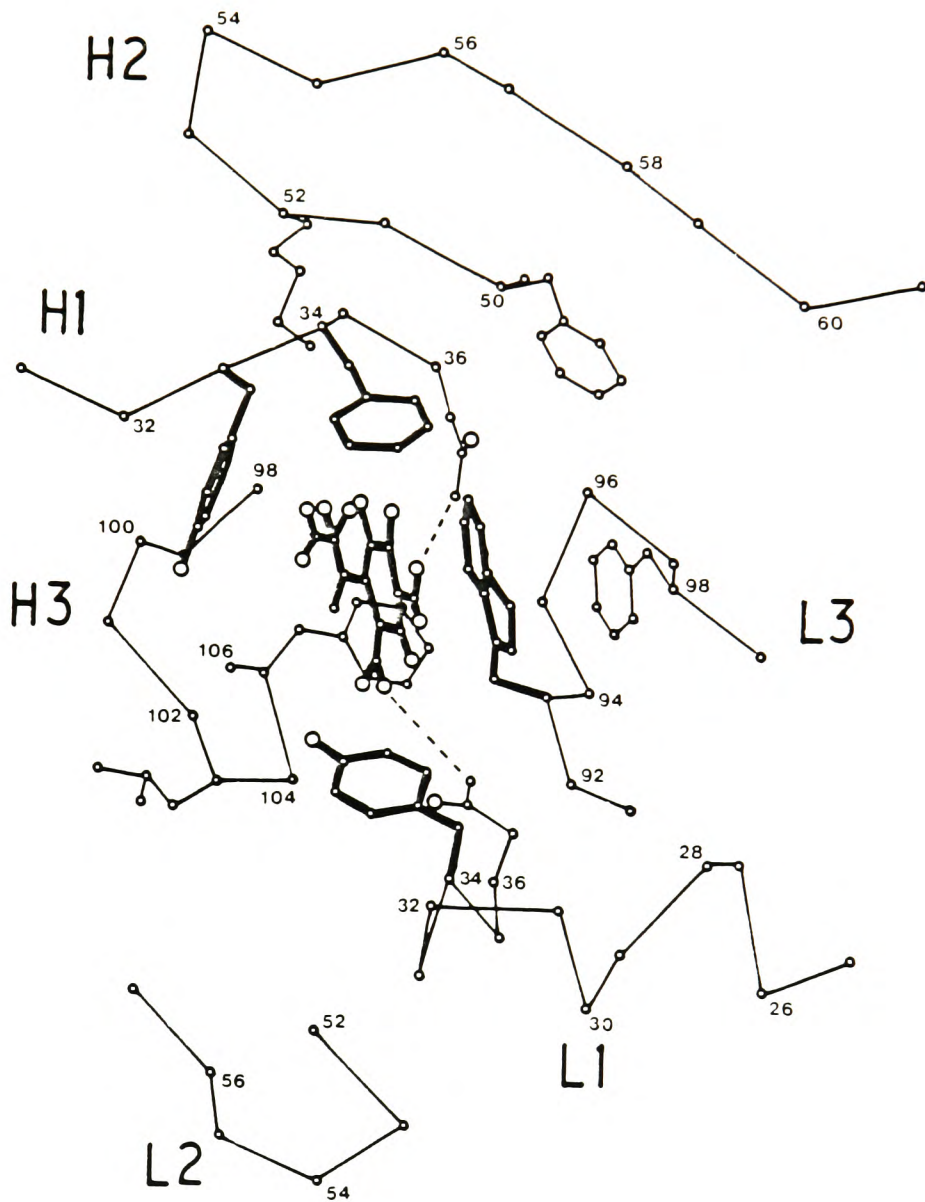


Figure 3.6 Postulated combining site of protein 315 containing DNP-glycine. The three hypervariable regions of the heavy (H) and light (L) chain are indicated. The haptens are orientated with the relative position of Trp-93_L and Phe-34_H as in Fig. 3.4b. Possible polar interactions (or hydrogen bonds) are indicated between the haptens and the side chains of Asn-36_L and Asn-36_H.

be arranged as Fig. 3.4b by minor alteration of the position of Phe-34_H, and by rotation of the side chain of Trp-93_L. The side chain of Tyr-34_L was moved further away from the framework to occupy a position compatible with the results of the previous chapter. Minor alterations were made to the position of the phenolic ring of Tyr-33_H so that the required shift of 1 ppm was obtained on the side chain of DNP-glycine. An additional result which may be included is that in Hardy & Richards (1978) of an upfield shift of 1 ppm on binding of the proton of a hapten p-CHF₂ group. The shifts from Trp-93_L and Phe-34_H (the nearest rings) would only account for < 0.5 ppm, so the presence of an additional aromatic ring at the back of the site can be inferred. The side chain of Phe-105_H can be positioned close enough to produce the required shift, while being too far from the hapten ring protons (> 0.6nm) to affect them significantly. During this re-building, care was taken not to deviate further from the original site of Padlan et al. (1976).

The final site is shown in Fig. 3.6 and the calculated ring-current shifts for this arrangement are given in Table 3.4. Also depicted in the figure are possible polar interactions (or H-bonds) to the side chains of Asn-36_L and Asn-36_H from the 2- and 4-nitro groups respectively (the evidence for such interactions was discussed in section 1.10.2). It is clear that this site provides a reasonable explanation of the shift changes on hapten binding (also see Fig. 3.5 and Table 3.3).

3.3.5 Probable accuracy of this predicted site

The chemical shift changes on haptens bound to protein 315 are sufficiently large that it is quite reasonable to assume that the major

Table 3.4

Comparison of calculated with experimental shift changes (ppm) on the protons of DNP-glycine using the co-ordinates of the final model in Fig. 3.6^a

Ring	Proton			
	(H3)	(H5)	(H6)	CH ₂
Trp-93 _L	1.08	0.60	1.24	0.26
Tyr-33 _H		0.01	0.17	0.56
Phe-34 _H		1.52	0.55	0.16
Phe-105 _H	0.17	0.13		
total	1.25	2.26	1.96	0.98
exptl ^b	1.21	2.20	1.77	1.00
calcd - exptl	+0.06	+0.06	+0.19	-0.02

^a Only aromatic rings $\leq 6 \text{ \AA}$ from the proton of interest were considered (Dwek, 1973)

^b From Dower et al. (1977)

Table 3.5

Comparison of the distance of Tyr-34_L from the first hypervariable residue of loops L1 and L3

Atom	Distance to Tyr-34 _L (Å)					
	REI		Mcg		M315 ^a	
	C ^α	O ^η	C ^α	O ^η	C ^α	O ^η
C ^α Asn-36 _L	6.9	12.8	6.3	12.2	6.6	11.8
C ^α Ala-91 _L	8.3	14.4	8.0	14.0	8.5	13.5

^a from the refined site depicted in Fig. 3.6. Compare this table with Table 2.4.

cause is ring-currents, i.e. that the observed shift is equal to the ring-current shift $\pm \sim 0.2$ ppm. Therefore if the available theory for predicting these shifts is adequate (discussed in section 3.1.3), the proposed site should closely reflect the actual site, providing the starting model of the site was reasonably accurate.

How well this refined site is in agreement with other experimental data is summarized below (the requirements for these criteria to be fulfilled were given in section 1.10.2).

Position of Tyr-34_L. A comparison of the predicted location of this residue with that found in proteins REI and Mcg is given in Table 3.5 (see section 2.3.4). The position of this residue relative to the framework is now approximately the same in all three structures, and is therefore now compatible with the requirements described in chapter 2.

Position of Tyr-33_H. This residue is positioned to provide the shift on hapten side chains. This location is also compatible with the nitration studies of the previous chapter.

Position of Lys-52_H. The side chain of this residue (Fig. 3.6) is sufficiently close to the hapten to be affinity labelled by BADL (Haimovich et al., 1970, 1972). It is also possible to cross-link it to Tyr-34_L using DIBAB (Givol et al., 1971).

Involvement of a light-chain tryptophanyl side chain. This is provided by Trp-93_L.

Provision of a positively-charged sub-site. The side chain of Lys-52_H is sufficiently close to provide this sub-site. Its presence is implicit from affinity labelling studies (Haimovich et al., 1970, 1972; Givol et al., 1971), although crude protection experiments (Klostergaard et al., 1977a,b) have suggested the presence of an arginyl rather than a lysyl side chain as the positively charged grouping. If this is the

case the side chain of Arg-95_L could fulfill this role, as was suggested by Dower et al. (1977), although the absence of a positive sub-site in the (V_L)₂ fragment (Gavish et al., 1977) favours the involvement of a heavy-chain residue.

Hydrogen bonds to the 2- and 4-nitro groups of DNP. The side chains of Asn-36_H and Asn-36_L are sufficiently close to provide hydrogen bonds if required (as in the original site proposed by Padlan et al., 1976).

Overall dimensions. The depth of the site is compatible with that found from spin-label studies (Sutton et al., 1977). This work also showed that the entrance of the site was narrower on one side than the other. This is accounted for by the presence of Tyr-33_H close to the side chain of the haptens. The other side of the site was found to be relatively open. These studies also showed that the positively-charged grouping was at the side of the site where motion is restricted, again possibly indicating a heavy-chain residue as the positive sub-site.

Multi-specificity. The shape of the site is such that a wide variety of ligands may be accommodated in a similar manner, while still accounting for the experimental chemical shift changes (see Fig. 3.5 and Table 3.3). It should be noted that with the site of Dower et al. (1977) it is not possible to account for the shift changes on DNNS. The binding energy for the various ligands probably comes mainly from non-polar interactions, and the exclusion of water (Janin & Chothia, 1978a,b).

Despite the satisfactory way that this site now accounts for the known experimental observations on the binding of haptens by protein 315, an unequivocal assessment of its accuracy can only be made when the crystallographic structure of the Fv fragment has been determined. This structural determination is currently underway (Aschaffenberg et al., 1979). The results are awaited with interest.

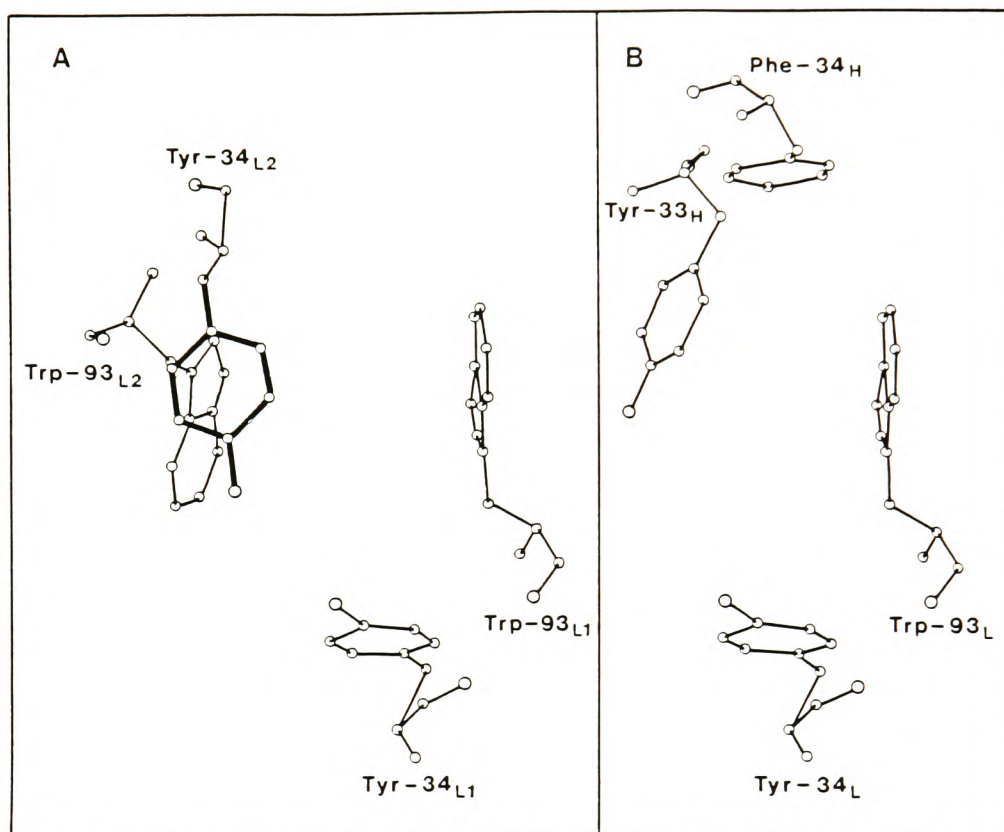


Figure 3.7 (a) The generated model of the combining site of the V_L dimer of protein 315. The model was constructed by using the co-ordinates of protein REI, as described in the text. (b) Combining site of the Fv fragment of protein 315 (as Fig. 3.6).

3.3.6 The binding of haptens to the light-chain dimer of M315

Any postulated site must also explain the continued binding of DNP haptens, albeit weakly, by the isolated light-chain dimer of M315 (Schechter et al., 1976; Gavish et al., 1977). A stacking interaction still occurs with a tryptophan side chain, as determined from CD studies (Freed et al., 1976) and UV difference spectroscopy (Gavish et al., 1978). Since the $^1\text{H-NMR}$ hapten-difference spectra for the Fv fragment and the V_L dimer show many similarities (Jackson et al., 1981), it is reasonable to presume that the overall structure of the L-chain hypervariable loops and their interactions with haptens remain basically the same in the Fv and V_L dimer. Therefore the major interaction is still with Trp-93_L. In view of this, a model of a hypothetical light-chain dimer was made from the proposed structure of the light-chain hypervariable loops, on the basis of the mode of dimerization of protein REI (as described in section 3.2.3). Since the construction of such a model relies on homologies between hypervariable loops, and since the mode of dimerization of V_L domains may not be invariant (Wang et al., 1979), the model must be regarded only as a qualitative aid to the interpretation of any studies on the V_L dimer.

The structure of the resultant site is compared with the proposed site of the whole protein in Fig. 3.7. The resultant site is highly aromatic, in agreement with $^1\text{H-NMR}$ studies (Jackson et al., 1981). The V_L dimer is divalent at pH 8, changing to monovalent at pH 6 (Gavish et al., 1977). This indicates that at the high pH a DNP ring can interact with each tryptophan, and suggests that the site alters size / shape over this pH range. The $^1\text{H-NMR}$ shifts on hapten protons of DNP-glycine and DNP-aspartate when bound to the V_L dimer are given in

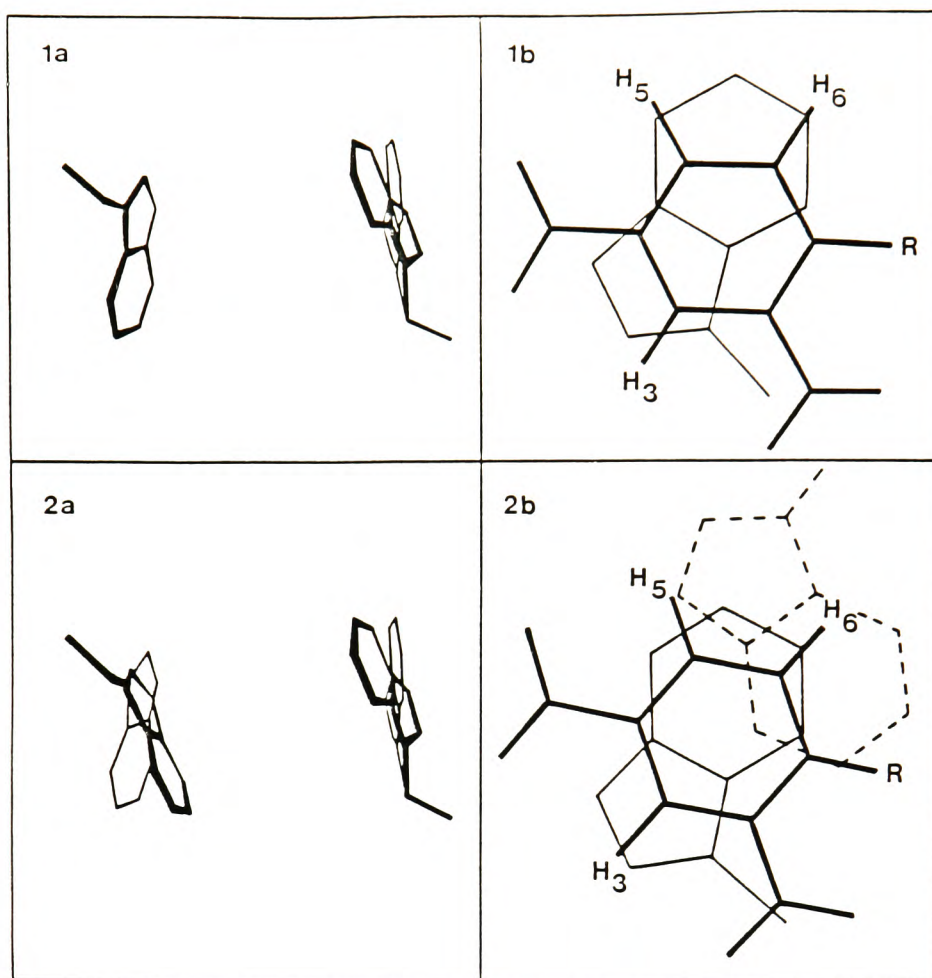


Figure 3.8 Possible geometries of the DNP rings relative to the two tryptophan residues in the combining site of the V_L dimer to account for the experimental shift changes (Table 3.6). (1) DNP-aspartate; (2) DNP-glycine. (a) Orientation of tryptophans. Lightly drawn residues indicate original positions as, in Fig. 3.7. Heavily drawn residues represent orientation after repositioning of side chains to explain chemical shifts on haptens (Table 3.6). (1b) Position of DNP-aspartate in the site 1a. The tryptophan ring of monomer 1 is parallel to and 0.33 nm from the DNP ring (tryptophan monomer 2 (not shown) is at an angle to the DNP ring). (2b) Position of DNP-glycine in site 2a. The ring of tryptophan monomer 1 is shown solid, with that of monomer 2 dashed. Both tryptophan rings are 0.33 nm from and parallel to the DNP ring.

Table 3.6. A large difference is found between these two haptens, in contrast to the similar shifts found with various DNP haptens bound to the Fv fragment (Dower et al., 1977). These shifts may be explained by the geometries in Fig. 3.8 (the calculated shifts are given in Table 3.6). The presence of two tryptophans allows the very large shifts to be accounted for, slightly differing interactions with the tryptophan of the second monomer would explain the lesser shift obtained with DNP-aspartate. The ability of this proposed arrangement to explain the experimental data is good support for the validity of the proposed light-chain hypervariable loop structures.

3.3.7 Antibody combining sites - conclusions

The site of protein 315 described above allows some information to be deduced about the nature of this antibody combining site. The cross-reactivity of the protein may be explained in terms of an essentially pre-formed site of defined structure - antigens of correct 'shape' will fit into this site and bind. More precisely stated, the antigen must have the correct overall stereoelectronic requisites for fit and interaction with the amino acid side chains comprising the site. Since the site of protein 315 is highly aromatic, slight variations in the position of different ligands in the site are reflected in relatively large changes in the fully-bound chemical shifts of the ligand protons. This is due to the sensitivity of ring-current shifts to the position of a proton relative to an aromatic grouping. The fully-bound shift changes for different DNP ligands binding to protein 315 are very similar (Dower et al., 1977), indicating that the positioning of these haptens in the site is almost identical (differences in position of the DNP ring of < 0.05 nm). It is

Table 3.6

Comparison of experimental and calculated^a chemical shift changes (ppm) of ligand proton resonances on binding to the V_L dimer

	calculated shift change						
	DNP-glycine				DNP-aspartate		
	H(3)	H(5)	H(6)	CH ₂	H(3)	H(5)	H(6)
Trp-93 _L (monomer 1)	1.55	0.95	0.80	0.05	1.10	1.30	1.05
Trp-93 _L (monomer 2)	0.10	1.65	2.40	1.10	0.30	0.40	0.25
total	1.65	2.60	3.2	1.15	1.40	1.70	1.30
	experimental shift change ^b						
	1.6	2.7	3.0	1.2	1.2	1.5	1.1

^a Using the geometries in Fig. 3.8

^b From Jackson et al. (1981)

interesting that the much weaker binding of DNP ligands to the V_L dimer of protein 315 is accompanied by a much larger variation in the fully-bound chemical shift changes of different haptens. This indicates that these ligands have a less well-defined set of interactions with the aromatic "walls" of the site. It is probable that this reflects a less than optimal size / shape / electronic environment for DNP ligands when the V_H domain is replaced by another V_L domain. This emphasizes the requirement not only for specific interactions (in this case the tryptophan side chain) but for the correct overall structure if a high binding constant is to be obtained.

3.4 SUMMARY

The suitability of ring-current calculations to refine the structure of antibody combining sites (using the revised ring-current intensity factors for tryptophan rings of Perkins & Dwek [1980]) is confirmed by the ability of such calculations, based on the known structure of protein 603, to account satisfactorily for the experimental changes in chemical shift of haptens bound to this antibody. Using the revised tryptophan ring-current intensity factors to calculate geometries consistent with the experimental chemical shift changes, a model of the combining site of protein 315 was built. This site is compatible with the available experimental evidence on this protein, and resolves the discrepancies in the position of Tyr-34_L which were described in the previous chapter. The resultant site allows the experimental shift changes of a wide variety of different haptens to be accounted for. The arrangement of light-chain residues in the site is also compatible with the binding studies on the V_L dimer (Jackson et al., 1981).

It is inferred that the antigenic specificity of protein 315 is essentially a question of 'shape'. Haptens of appropriate shape / electronic configuration bind to the site, with the tightest binding ligands being those which 'fit' the site best.

CHAPTER 4

An Assay For Clq Binding Which Allows Quantitative Comparison of Different Immunoglobulins

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4.1 INTRODUCTION

4.1.1 Requirement for a Clq binding assay

As was discussed in section 1.6, the ability of antibody-antigen complexes to bind the Clq subcomponent of the Cl macromolecular complex is one of the most important secondary functions of the antibody molecule. As part of this thesis examines the role of the conserved C_H2 domain oligosaccharide of IgG by studying the properties of aglycosyl IgG, it is important to ascertain the possible involvement of this oligosaccharide in Clq binding. This necessarily involves a comparison of the Clq binding abilities of native and aglycosyl IgGs, and therefore requires an assay whereby such a comparison may be quantitatively made. In the absence of a satisfactory assay system (as is discussed below), this chapter describes the development of a suitable assay. The resultant assay is used in chapter 6 to test the binding of Clq to aglycosyl IgG.

4.1.2 Assays for Clq binding

In order to define a binding curve it is necessary to determine the amounts of a species bound and free at several different concentrations. In the simplest case of a single dissociation constant and no co-operative effects the following equation applies:

$$B = \frac{\text{Cap} \cdot F}{K_d + F} \quad (4.1)$$

B = Concentration of species bound

F = Concentration of species free

Kd = Dissociation constant

Cap = Capacity

In Clq binding assays the bound Clq is generally separated from the free by having the IgG to which it binds insolubilized in some manner - so that the IgG, together with any bound Clq, may be separated from free Clq by centrifugation. If the Clq is ^{125}I -labelled then the amount in each fraction is easily determined.

The binding of Clq to monomeric IgG is weak ($K_d \sim 10^{-4}$ M: Sledge & Bing, 1973; Schumaker et al., 1976) and therefore direct binding / inhibition requires high concentrations. Aggregated IgG (by immune or non-immune methods) binds Clq with a K_d of $\sim 10^{-8}$ M (Lin & Fletcher, 1978; Hughes-Jones, 1977; Emanuel et al., 1982a), thus allowing proportionately less IgG to be used. Since this is preferable for the experiments described in chapter 6, only assay systems which utilize aggregated IgG are considered.

The following types of IgG complexes have been previously used to study Clq binding :

1. Antibodies aggregated by chemical cross-linking (Lin & Fletcher, 1978).
2. Antibodies aggregated by precipitation using organic solvent (Allan & Isliker, 1974b).
3. Covalently linked IgG oligomers of defined size (Jones et al., 1979; Tschopp et al., 1980a,c; Wright et al., 1980a,b).
4. Antibody/antigen immune aggregates (Heusser et al., 1973; Goers & Porter, 1978; Burton et al., 1980; Emanuel et al., 1982a,b).
5. Antibody/red blood cell immune complexes (Volanakis & Stroud, 1973;

Goers & Porter, 1978).

The first two methods are non-physiological and so do not require immune IgG. Although useful information can be obtained using these systems, its relevance may be questioned. For example, with chemically cross-linked IgG it has been shown that although Clq binds to the complexes, C1 is not activated (Folkerd et al., 1980). It is therefore advisable that such systems are avoided if an immune system is available.

Defined IgG oligomers (dimers, trimers etc.) bind Clq much more tightly than does the monomeric species ($K_d = 10^6 \text{ M}^{-1}$ for dimers, 10^7 M^{-1} for trimers and $3 \times 10^9 \text{ M}^{-1}$ for tetramers [Wright et al., 1980b]), and therefore make useful model immune complexes. Although direct binding studies may be performed (Tschopp et al., 1980c), on a routine basis their use as inhibitors would be more practicable as they do not precipitate Clq. The disadvantages of such a system for the requirements described in section 4.1.1 are twofold. Firstly the procedure of chemically cross-linking is not ideally suited to making two identical preparations from different IgGs. Secondly, and more importantly, the yield of products is not high - necessitating large amounts of starting material. Since only small quantities of aglycosyl IgG are to be available this makes the use of defined oligomers impracticable.

Immune aggregates offer a more natural system, with the characteristic antibody/antigen precipitate being used to bind Clq. Although this binding assay is straightforward when using a single source of aggregates, some difficulties occur when comparing different antibody populations. Firstly, Clq binding must be tested at some point

(usually equivalence) along the precipitin curve. Precipitin curves for different antibodies vary (as is discussed below), and Clq binding is dependent upon where on the curve the precipitates are formed (S. B. Easterbrook-Smith & R. A. Dwek, unpublished data). Secondly, this system requires a precipitating antibody-antigen complex which does not always occur (Terres, 1975), particularly with monoclonal antibodies. Thirdly, some derivatized proteins used as antigens interact with Clq. This is best characterised in the case of DNP-proteins (Loos & König, 1977), which are commonly used in immunological studies. These DNP-proteins are therefore unsuitable for forming immune complexes to use in Clq binding assays.

Antibody/red blood cell conjugates do not require the calculation of a precipitin curve providing that the antibody is in excess, as under such conditions all available sites will be occupied allowing unbound immunoglobulin to be separated by washing. This is an advantage when comparing different antibodies because at saturation there will be the same antibody to antigen ratio. The disadvantages of this system are as follows: the red cells are fragile and derivatization with non-red blood cell proteins / haptenic groups is difficult to achieve without lysis; such derivatization is difficult to reproduce between batches, and long term storage of the cells is not possible; dinitrophenylation again produces DNP-protein which interacts with Clq, making assays using anti-DNP antibodies difficult to quantify. The advantages of this system over antibody/antigen complexes are: the antibody need not be precipitating; the derivatized antigenic matrix is available without bound antibody as a control for non-specific binding (the antigen alone from the antibody/antigen system is soluble, so unless Clq precipitates it non-specific binding cannot be tested for); the antibody coats the

cell to give effectively a monolayer. This final point is a fundamental difference between these systems. In recent years it has been established that antibody/antigen precipitates do not form solely in terms of an 'infinite lattice', but that the Fc region plays a crucial role in their formation by contributing non-immune-specific hydrophobic interactions which aid the formation of large complexes (Steensgaard & Frich, 1979; Møller, 1979; Møller & Steensgaard, 1979; Jacobsen & Steensgaard, 1979; Rodwell et al., 1980). Modification of IgG has been shown to affect precipitin ability by interfering with these interactions (Jacobsen & Steensgaard, 1979), and it is therefore to be expected that different IgGs, by virtue of differing subclass or artificial modification, will potentially differ in the manner of their precipitation. This has important consequences for Clq binding, because lattice differences may lead to a change in the apparent binding constant (for example by altering the relative orientations of the Fc regions) without altering the intrinsic (i.e. free solution) binding constant. Also, since the large size of the Clq molecule limits its interaction to the surface IgG of the 3-dimensional antibody/antigen lattice, alterations in the average particle size of the precipitate will alter apparent capacity. This is probably the reason for the reduced capacity of antibody/antigen aggregates when compared with antibody/red blood cells (Goers & Porter, 1978), and potentially explains capacity changes observed after chemical modification of IgG (Burton et al., 1980; Emanuel et al., 1982a)

In conclusion, the influence of lattice effects on Clq binding makes results obtained using the antibody/antigen aggregate system potentially ambiguous. This is particularly the case when small changes in Kd or capacity are involved, and especially since most studies

(Burton et al., 1980; Winkelhake et al., 1980; Emanuel et al., 1982a,b) only compare precipitin curves up to equivalence - assuming that this is totally sufficient to detect changes in the nature of precipitation. However, even when differences between two IgGs occur, the amount of precipitate in this region is the same for each IgG (providing that binding of antigen is not affected), although differences are reflected in the time-dependance and turbidity development of the precipitates (Jacobsen & Steensgaard, 1979). It is only after equivalence that the amount of precipitate is altered. In contrast the immunoglobulin coated cell system allows a comparison of the binding ability of different Igs to be assessed in isolation, without the complications of the effects of lattice structure formation. This type of system is therefore preferable if the Clq binding ability of differing immunoglobulins is to be quantitatively compared.

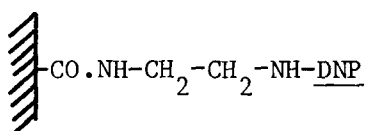
4.1.3 Development of an assay suitable for IgG-comparison

The assay developed below uses an antigen coupled support matrix to bind IgG, which is then used in a manner analogous to IgG coated cells. All the factors in favour of using IgG coated cells are retained, while, providing the support matrix fulfils certain requirements, the disadvantages are removed. In this respect an ideal support should :

1. Be easily and reproducably derivatized.
2. Be impermeable to molecules of high molecular weight (to give a 2-dimensional surface).
3. Stay in suspension long enough to allow equilibrium to be achieved, yet be easily pelleted by centrifugation.
4. Be sufficiently small to have a high surface area/volume ratio.

5. Not interact with Clq when derivatized.

A matrix which satisfies the above requirements is found in Affigel 701/702 (Bio-Rad). These are small ($2 \pm 1 \mu\text{m}$ diameter) derivatized polyacrylamide gel beads, with an exclusion volume of 10 kDaltons. Since DNP was to be used as the haptenic group, Affigel 701 was chosen as this is an aminoethyl derivatized support, and is therefore easily reacted with reagents such as dinitrobenzene sulphonic acid to produce



which provides DNP coupled to the support via a neutral spacer group. The experimental treatment of the assay is described in section 4.2.

This assay system is tested on mouse monoclonal IgGs of differing subclass to demonstrate its usefulness for comparative studies involving different IgGs. It is then utilized in chapter 6 to compare the Clq binding of glycosyl and aglycosyl IgG.

Table 4.1

Details of the cell lines used in these experiments (B. A. Askonas, personal communication):

Line	H chain	Fusion Line	Parent	Immunogen
A3	γ 1	P3-NSI/1-Ag4-1 ^c	CBA Spleen	DNP-KLH ^a
K3	γ 2a	P3-X63-Ag8.653 ^d	CBA Spleen	DNP-HGG ^b
K1	γ 2b	P3-X63-Ag8.653	CBA Spleen	DNP-HGG

^a KLH = Keyhole limpet haemocyanin

^b HGG = Human gamma globulin

^c Köhler et al., 1976

^d Kearney et al., 1979

4.2 MATERIALS AND METHODS

4.2.1 Preparation of Clq

Although the use of homologous Clq is to be preferred, the low availability of mouse serum makes preparation of mouse Clq prohibitive (although its purification has been achieved, and its properties found to be similar to human Clq [McManus & Nakane, 1980; Yonemasu & Sasaki, 1981]). Therefore human Clq was used, which was prepared from human serum and iodinated using Enzymeobeads (Bio-Rad) to < 0.05 I/Clq essentially as described by Tenner et al. (1981). At these levels of iodination the Clq remains fully active (Lin & Fletcher, 1978; Tenner et al., 1981). Purity was verified by SDS-PAGE (Laemmli, 1970; Reid, 1972), by analytical ultracentrifugation where the Clq ran as a single 10 S component (Schumaker, 1981), and immunodiffusion where a single fused precipitin arc was formed against anti-human Clq (Behringwerke) and anti-whole human serum (Miles). The molecular weight of Clq was taken to be 460,000 daltons (K. B. M. Reid, personal communication) with an extinction coefficient of $E_{280}^{1\%} = 6.82$ (Reid et al., 1972).

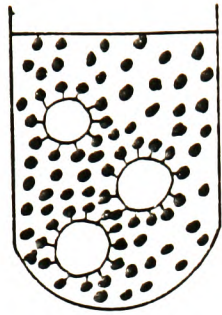
4.2.2 Hybridomas



The following hybridoma cell lines were produced by Dr. M. Kennedy and generously made available by Dr B. A. Askonas (both of N.I.M.R., Mill Hill): A3, IgG1; K3, IgG2a; K1, IgG2b. All three lines were raised by immunization of CBA mice with DNP derivatized protein, followed by fusion of the spleen cells with non-secreting plasmacytoma cells (Köhler & Milstein 1975, 1976). Full details of these lines are given in Table 4.1. The hybridomas were maintained as ascitic tumours by

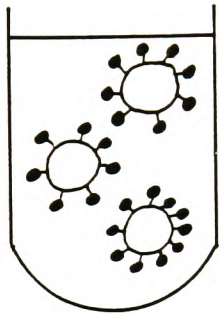
serial passage in CBA x Balb/c F₁ mice injected intraperitoneally with 0.5 ml of pristane at least two weeks previously. The mice were injected with 1-2 x 10⁶ cells in 0.2-0.5 ml of BSS[-Ca, -Mg]. After approximately two weeks the ascitic fluid was aspirated from the peritoneal cavity and allowed to clot. The supernatant after centrifugation was stored at -20°C until required. IgG was isolated from this ascitic fluid (after dialysis against PBS / EDTA) on a DNP-lysine-Sepharose affinity column equilibrated in PBS / EDTA, and eluted with 50 mM DNP-glycine pH 7.2. The DNP-glycine was removed by Dowex 1X8-400 (Sigma) chromatography in PBS, except for K3 protein where this was not possible due to the extremely high binding constant of this protein for the hapten. In this case the DNP-glycine was displaced by exchange dialysis against 0.1 M dinitrophenol / 0.1 M tris-HCl pH 7.4 (Eisen, 1964a). The dinitrophenol could then be removed on a column of Dowex 1X8-400 as above. Purity of the proteins was verified by SDS-PAGE (Laemmli, 1970), and the identity of the subclasses confirmed by their elution profiles from SpA-Sepharose (Ey et al., 1978). Periodically the IgG produced was analysed by SDS-PAGE to confirm that it was the same as that produced by the line originally, as reversion / mutation of such cell lines is not uncommon.

4.2.3 Preparation of DNP-Affigel 701

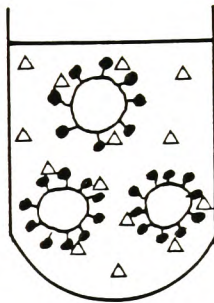
Affigel 701 was reacted as a 10% suspension with 30 mM DNBS (Eastman-Kodak, recrystallised as described by Eisen, 1964a) in 0.2 M Na₂CO₃ at 37°C in the dark for two days to ensure complete reaction. Excess reagents were removed by dialysis. The DNP-Affigel 701 is stable in PBS / 0.02% NaN₃ at 4°C in the dark for at least a year. The volume % of Affigel was estimated by centrifugation of a suspension in




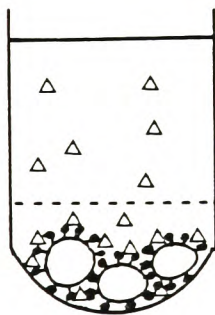
1. Incubate DNP-Affigel 701 () with excess antibody ()



2. Wash



3. Incubate with ^{125}I -C1q ()



4. Separate free and bound C1q by centrifugation

C1q Binding Assay

Figure 4.1 Diagrammatic representation of the C1q binding assay.

haematocrit capillaries.

4.2.4 Clq binding assay

DNP-Affigel 701 was incubated with excess IgG for 1 hour at room temperature followed by 1 hour at 4°C. Unbound excess IgG was removed by four cycles of centrifugation followed by resuspension in PBS. No significant dissociation of the bound IgG occurred under these conditions with the monoclonal antibodies studied. In preliminary experiments it was found that IgG/DNP-Affigel at a final concentration of 0.3% (v/v) gave a suitable capacity for Clq under the conditions used. The capacity of DNP-Affigel for IgG is approximately 7-8 mg per ml of gel (results not shown). The stock solution of [¹²⁵I]-Clq was diluted to an appropriate specific activity with unlabelled Clq, made 1% (w/v) with BSA, and then centrifuged at 10,000 x g for 10 minutes in an Eppendorf centrifuge before use to remove any precipitable material. Aliquots of the supernatant were added to 100 µl of a 0.6% suspension of IgG/DNP-Affigel / PBS in Luckham LP/2 tubes, and the volume made up to 200 µl with PBS / 1% BSA. The final conditions are: Clq, 0 to ~ 70 nM; BSA, 0.5%; IgG/DNP-Affigel, 0.3%; total volume, 200 µl. The tubes were then thoroughly mixed, and incubated at 37°C for 45 minutes to allow equilibrium to be reached (Emanuel et al., 1982a). After this time the IgG/DNP-Affigel together with any bound Clq was pelleted by centrifugation for 1-2 minutes in an Eppendorf centrifuge. A fixed aliquot of the supernatant (usually 160 µl) was removed, and this fraction as well as the remaining pellet fraction were counted in an LKB gamma counter to determine the amount of Clq in the supernatant (S), and in the pellet (P). This is shown diagrammatically in Fig. 4.1. At least five control tubes were also included which were treated

identically to an experimental tube except that 100 μ l of PBS was substituted for the Affigel suspension. If any precipitation of Clq was observed in these controls the experiment was discarded.

4.2.5 Calculation of Clq bound and Clq free

It follows from the way that the supernatant and pellet fractions are separated that if P and S represent amounts of Clq in the pellet and supernatant, and V_p and V_s are the volumes of these fractions respectively then:

$$\text{Clq bound, } B = P - \frac{V_p}{V_s} \cdot S \quad (4.2)$$

$$\text{Clq free, } F = S \cdot \frac{V_s + V_p}{V_s} \quad (4.3)$$

The most accurate determination of the fraction volumes would involve the use of an internal volume marker. For the purposes of this experiment [^{131}I]-BSA would be ideal as it would give directly the volume available to the Clq, and it is this technique which is recommended. If dual-channel γ -counting facilities are not available it is possible to calculate the average volume of the pellet and supernatant fractions from the control tubes. It is this alternative which was used. Using 'c' and 'e' to denote control and experimental respectively, then equations 4.2 and 4.3 become:

$$\text{Clq bound, } B = P_e - \left(\frac{P_c}{S_c} \right) \cdot S_e \quad (4.4)$$

$$\text{Clq free, } F = S_e \cdot \left(\frac{S_c + P_c}{S_c} \right) \quad (4.5)$$

where $\overline{\left(\frac{Pc}{Sc}\right)}$ denotes the mean of $\frac{Pc}{Sc}$ and $\overline{\left(\frac{Sc + Pc}{Sc}\right)}$ the mean of $\frac{Sc + Pc}{Sc}$.

4.2.6 Analysis of binding curves

The experimental data were fitted to equation 4.1 by non-linear regression using the FORTRAN 77 program BIND (appendix 1) to yield best-fit values for Kd and capacity. These parameters are written in the text ± their standard errors, which are also obtained from the program.

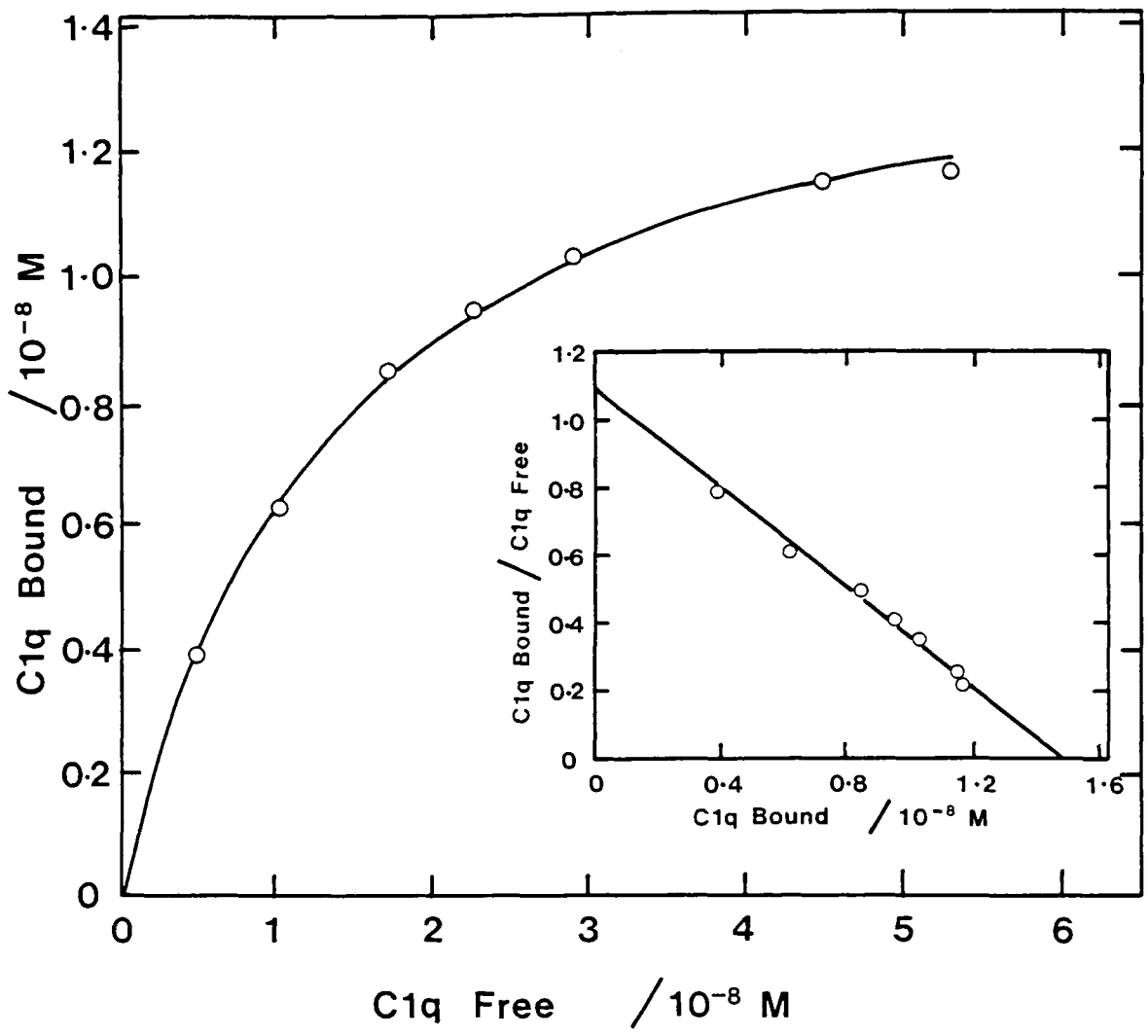


Figure 4.2 Binding of [^{125}I]-C1q to IgG2a-DNP-Affigel 701 at 37°C in PBS.

4.3 RESULTS AND DISCUSSION

4.3.1 Binding of Clq to mouse IgG/DNP-Affigel 701

The binding curve obtained with IgG2a is shown in Fig. 4.2. The values for the best-fit curve are $K_d = 1.33 \pm 0.08 \times 10^{-8}$ M and Capacity = $1.48 \pm 0.03 \times 10^{-8}$ M. This is approximately the same as previously reported values for the binding constant of Clq to IgG aggregates (the exact value is highly dependant on the ionic strength), (Hughes-Jones, 1977; Lin & Fletcher, 1978; Emanuel et al., 1982a). This capacity represents approximately 1 molecule of Clq per 9 molecules of IgG. No deviations from a simple hyperbolic curve are detectable, as is illustrated by the Scatchard plot being linear over the range covered, justifying the simple model chosen to fit the data (equation 4.1).

In order to establish that the binding is physiological it is necessary to show that: 1) no non-specific binding occurs; 2) the association constant and capacity are in the range expected for the process; and 3) the subclass specificity of Clq binding occurs. Fig. 4.3 shows the binding of [¹²⁵I]-Clq to DNP-Affigel 701 alone, and to DNP-Affigel 701 bound to IgG1, IgG2a, and IgG2b (the remaining mouse subclass IgG3 is less common and was not available for testing). The results are summarized in Table 4.2. No binding is observed to DNP-Affigel 701 alone over the concentration range of Clq studied, establishing the usefulness of this support. The non-specific binding of Clq to DNP-proteins is probably due to the dinitrophenylated protein being highly negatively charged, as its reacted lysine groups become neutral after dinitrophenylation. Since Clq has a pI of 10.0-10.6 (Lin & Fletcher, 1978) it will tend to bind ionically. DNP-Affigel 701 in

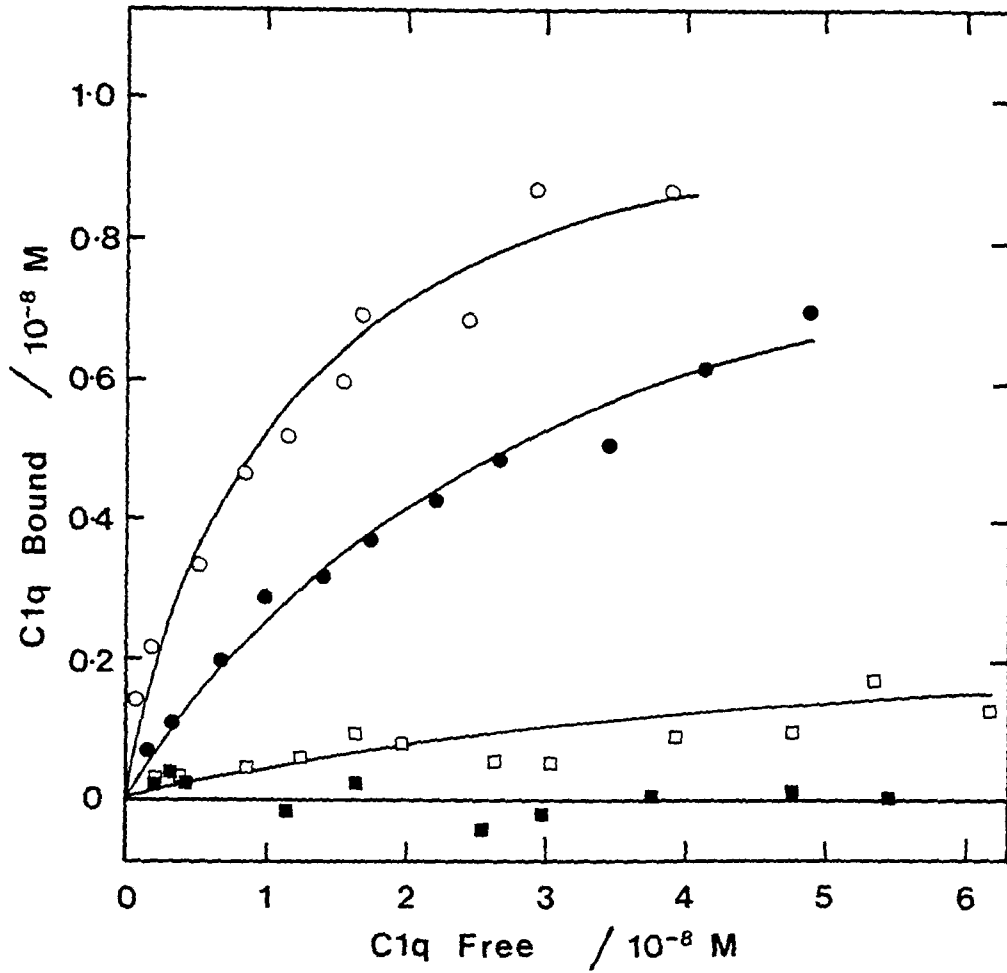


Figure 4.3 Binding of [¹²⁵I]-C1q at 37°C in PBS to:

- a) DNP-Affigel 701 (■)
- b) IgG1-DNP-Affigel 701 (□)
- c) IgG2a-DNP-Affigel 701 (○)
- d) IgG2b-DNP-Affigel 701 (●)

Table 4.2

Best fit values for K_d and capacity (obtained using program BIND) for binding of [125 I]-Clq to mouse IgG1, IgG2a, and IgG2b bound to DNP-Affigel 701 (from Fig. 4.3).

Subclass	K_d	S.E.	Capacity	S.E.
	<hr/>		<hr/>	
	/10 ⁻⁸ M		/10 ⁻⁸ M	
1	$\geq 25^a$			
2a	1.13	0.28	1.12	0.10
2b	3.32	0.61	1.12	0.11

^a Calculated assuming the same value for capacity as is found with IgG2a and IgG2b.

contrast is neutral, and is shown not to bind Clq. Both mouse IgG2 subclasses are found to bind Clq. The capacity for these subclasses is the same, however the value of Kd for IgG2b is three times greater than that for IgG2a. It is not known whether this reflects a true difference, or the result of using human rather than mouse Clq. IgG1 binds Clq poorly, but still significantly above background. The curve is too ill-defined to allow an accurate estimate of Kd and capacity to be made, but if the capacity is assumed to be the same as the other two subclasses then the Kd may be estimated to be $\geq 25 \times 10^{-8}$ M.

These results correspond well with previous work on complement fixation by mouse subclasses where IgG2a and IgG2b fix well, while IgG1 fixes weakly or not at all (Neuberger & Rajewsky, 1981; Hirayama et al., 1982). It should be noted that some IgG1 proteins are found which do fix complement (Ey et al., 1979; Hirayama et al., 1982), the reason for this is not known although it could be due to the presence of two mouse IgG1 subclasses (Stanislowski & Mitard, 1976). A very recent report also finds that mouse IgG1 binds C1 weakly (Okada et al., 1983).

4.3.2 Suitability of this assay for IgG-comparison

In use the assay is found to be easy to perform, and to give reproducible values for the experimental parameters. As a normal binding assay it compares most favourably with those mentioned in section 4.1.2, and has the advantage that the IgG complexes used are produced more quickly and easily. Comparison of different IgGs involves no extra steps. Since the different IgGs used in the previous section demonstrated equivalent capacities for Clq this implies that the assay meets the requirements of section 4.1. The binding curves show no

deviations from the simple hyperbolic curve described in equation 4.1. This is advantageous as it is therefore possible to define the experimental curves in terms of just two parameters, which are both physically meaningful. This is also found with Clq binding to immune aggregates (Burton et al., 1980; Emanuel et al., 1982a,b) and IgM-Sepharose (Sledge & Bing, 1973), but not to chemically cross-linked IgG (Lin & Fletcher, 1978) where non-hyperbolic curves are obtained. Finally, this assay has the distinct advantage of allowing anti-DNP antibodies to be used without the risk of non-specific binding by Clq (or C1 - see chapter 6).

4.4 SUMMARY

A Clq binding assay is presented which is suitable for use in comparison of the binding ability of different antibodies, and which allows the quantitative determination of their binding constants. No non-specific binding is observed to a DNP-derivatized support, allowing the use of anti-DNP antibodies. Since it is relatively simple to couple any antigen to Affigel, this assay system should be generally applicable to any antibody-antigen system. Specifically, it makes possible a meaningful quantitative comparison of Clq binding to glycosyl and aglycosyl IgG. This comparison is described in chapter 6.

CHAPTER 5

Preparation in Cell Culture, and Purification of Aglycosyl Mouse IgG

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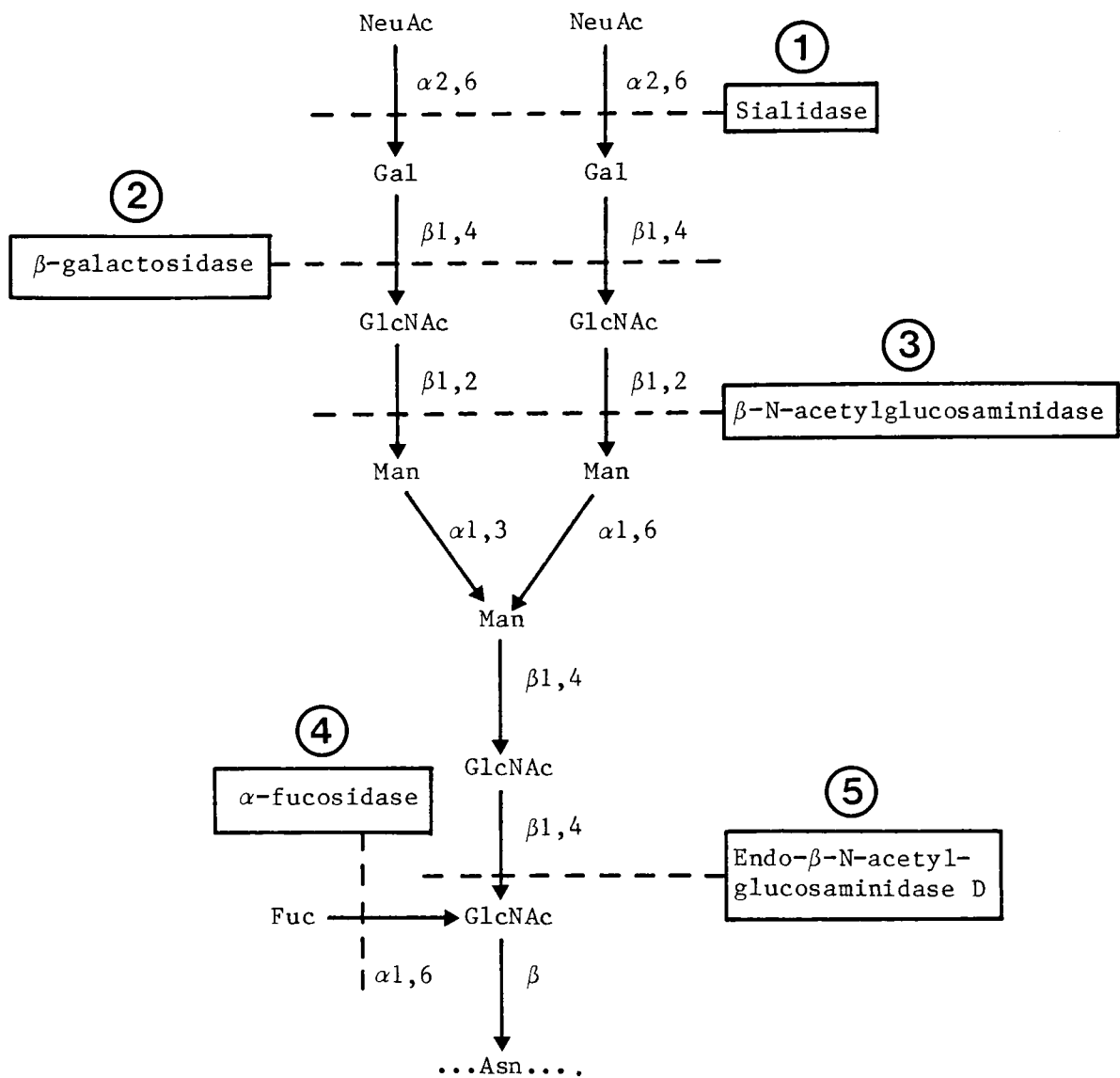
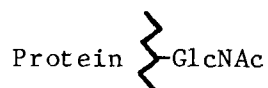


Figure 5.1 Removal of carbohydrate by sequential digestion of a representative complex-type oligosaccharide by glycosidase treatment.

step by digestion with endoglycosidase H from Streptomyces griseus, which cleaves the oligosaccharide between the first and second GlcNAc residues to leave:



For complex-type oligosaccharides such cleavage cannot be achieved with a single enzyme, and it is necessary to sequentially remove monosaccharide units by exoglycosidase action to produce a structure on which an endoglycosidase can act. The type of scheme which would be involved for a representative complex-type structure is shown in Fig. 5.1. Total digestion again leaves the same structure as above i.e. protein-GlcNAc. This assumes complete efficiency at every step, however since the process is necessarily sequential, incomplete digestion at one point prevents the action of subsequent glycosidases. In practice this means that the end products will almost invariably be a mixture of semi-complete chains. Longer digestion increases efficiency, but the glycosidase preparations must be free of extraneous protease activity which can be difficult as the sources of these enzymes often contain substantial amounts of protease. This is an important point as removal of carbohydrate often leads to increased proteolytic susceptibility (see section 6.1.2).

One advantage of sequential glycosidase treatment is that it is possible, providing that each enzyme is available separately, to produce proteins in various stages of deglycosylation. These may then be used to determine at what stage any functional defect becomes apparent. This approach has been used to show the importance of galactose exposure in regulating the catabolic rate of serum glycoproteins (see section 6.1.1). Also in studies on deglycosylation of IgG it has been shown that no difference is detected on removal of peripheral sugar residues

whereas functional differences were observed on further removal of core residues (Koide et al., 1977).

Although the glycosidase digestion schemes given above all leave the protein-GlcNAc linkage intact, Winkelhake et al. (1980) have described the use of an endoglycosidase which was claimed to remove entire complex-type oligosaccharides from an intact protein in a single step. Moreover this was achieved with IgG, and would therefore appear to be an extremely good method for production of deglycosyl IgG. The enzyme used was β -aspartyl N-acetylglucosaminyl amidohydrolase from hen oviduct, isolated as described by Tarentino & Maley (1969). However, in this latter paper the authors clearly show that the specificity of their enzyme is such that for the Asn-GlcNAc linkage to be cleaved the Asn must possess both free amino and carboxy termini; addition of amino acid(s) at either end totally prevents activity. This specificity is in keeping with its location in the lysosomes where, together with the many proteases present, it is thought to play a degradative role. In view of this it is unlikely that it could act on an Asn-GlcNAc linkage in a whole protein, and therefore claims of deglycosylation of intact IgG by this method must be viewed with caution.

Although the above enzyme cannot act on glycopeptides, an amidase which does cleave such β -aspartylglycosyl linkages has been isolated from almond emulsin (Takahashi, 1977; Takahashi & Nishibe, 1978, 1981). This almond glycopeptidase has been used to release carbohydrate from whole protein for functional studies (Nishibe & Takahashi, 1981; Takahashi et al., 1982). However the removal of carbohydrate from the native glycoprotein is much slower than removal from glycopeptides, and consequently the yield of deglycosyl protein is low. For example only

40% of the sugars could be removed from desialylated fibrinogen (Nishibe & Takahashi, 1981), with Taka-amylase A the yield of deglycosylated protein was only 10% (Takahashi et al., 1981), and the Fab_μ fragment from IgM was completely resistant (Tarentino & Plummer, 1982). Therefore a purification step is necessary to separate the deglycosyl from the glycosyl species. If this can be performed e.g. by use of immobilized lectins, then use of this enzyme is a good means of obtaining deglycosylated proteins. An alternative procedure is to digest the glycoprotein in the presence of chaotropic reagents which increase the efficiency of the digestion, suggesting that the different rates observed are due to the tertiary structure of the protein restricting access to the site of hydrolysis (Tarentino & Plummer, 1982). However, the use of chaotropic agents detracts from the mildness of the enzymic approach, and could cause permanent denaturation.

b) Chemical The specific removal of carbohydrate by chemical means is attractive, as it does not involve lengthy purification of glycosidases nor require any knowledge of the carbohydrate sequence. Two chemical treatments have been employed for this purpose, hydrogen fluoride (HF) (Mort & Lamport, 1977) and trifluoromethane sulphonic acid (TMFS) (Edge et al., 1981). Both reactions are performed in anhydrous conditions, with anisole generally added as a scavenger (see Mort & Lamport, 1977). In both cases only O-glycosidic linkages are attacked, and so the first GlcNAc residue of an N-linked oligosaccharide remains attached to the protein. Although HF has been most widely used, the more recent TMFS procedure has the advantages of being less hazardous and not requiring any special apparatus. The disadvantage of these procedures for general usage is that they involve potentially denaturing non-aqueous steps and extremes of pH. This means that it is dangerous to ascribe any

differences detected on deglycosylation to the mere lack of sugar residues. These techniques are probably most useful on small proteins which may be expected to refold after denaturation, and not IgG where the extremes of pH alone would be expected to seriously affect secondary function (Stollar et al., 1976; Winkelhake et al., 1980).

An inherent limitation of deglycosylation is that any conformational change in the protein which was caused by addition of carbohydrate will not necessarily be reversed after its removal.

5.1.3 Aglycosylation of glycoproteins

Various substances have been found to inhibit the glycosylation of proteins during biosynthesis and have therefore been widely used in studies on both the biosynthetic and functional aspects of protein glycosylation. The following is a brief summary of the types of inhibitor found. More extensive reviews may be found in Struck & Lennarz (1980) and Schwartz & Datema (1980, 1982).

1. Sugar analogues Various sugar analogues have been found to interfere with protein glycosylation. Of these the most widely used is 2-deoxy-D-glucose which is an analogue of glucose and (probably more significantly) mannose. It is not clear whether it blocks glycosylation as such, or acts as a 'chain terminator' after incorporation. 2-deoxy-D-glucose may also act by interfering with normal sugar metabolism, and the overall effect of deoxysugars on glycoprotein biosynthesis is probably due to a combination of a general inhibition of protein synthesis, catabolite repression or induction, and an inhibition of glycosylation. Fluorodeoxy sugars are also found to inhibit

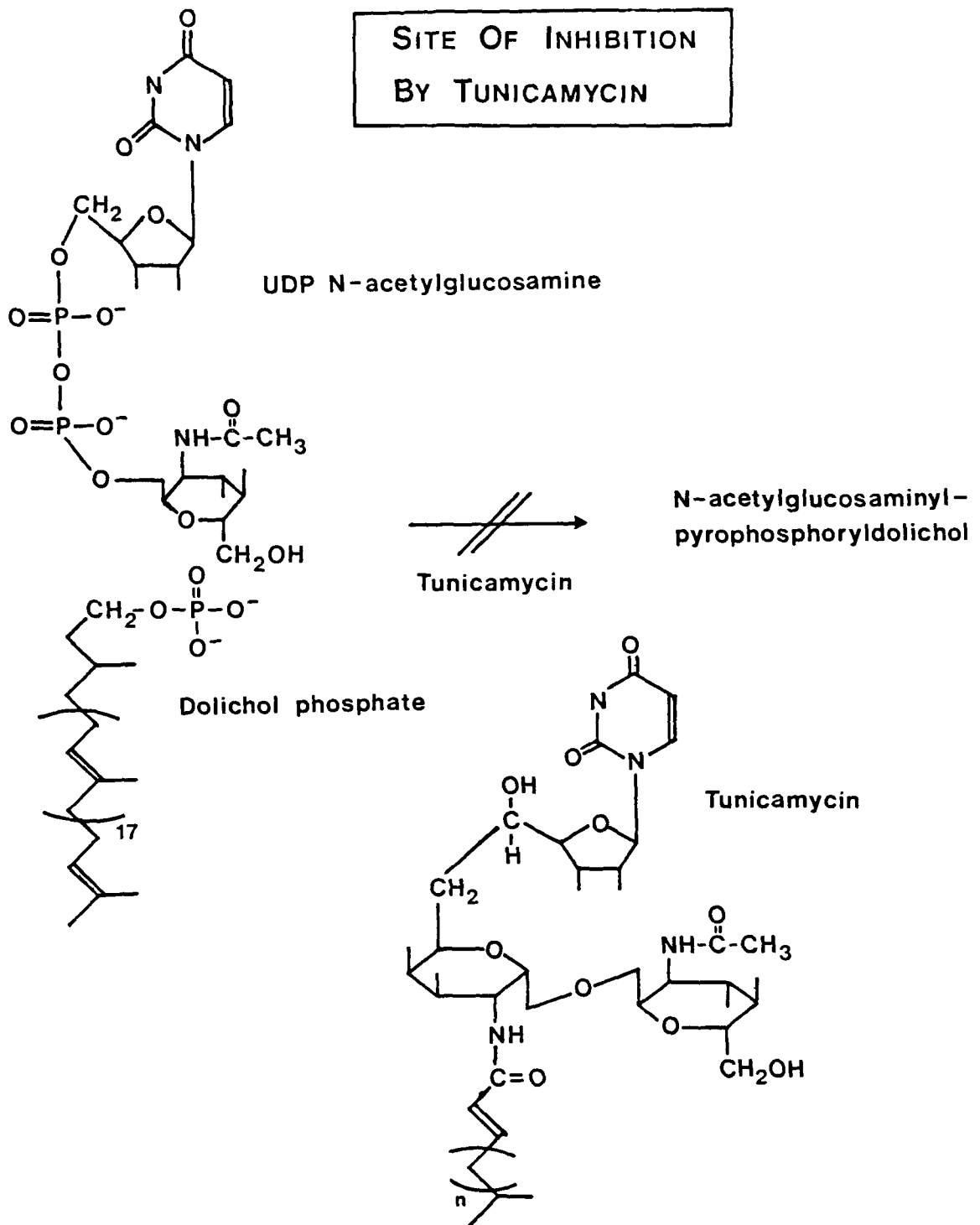


Figure 5.2 The structure and site of inhibition of tunicamycin. $n = 8$ to 11 (Takatsuki *et al.*, 1977).

glycosylation, and could be more useful than normal deoxysugars in that they are less likely to be incorporated into biosynthetic products.

2. Amino sugars Glycosylation is reversibly blocked by high concentrations of amino sugars such as glucosamine, although the block is not complete. Thus glycoproteins synthesized in the presence of a high glucosamine concentration may contain incomplete or abnormal carbohydrates.

3. Tunicamycin Tunicamycin is a potent inhibitor of protein glycosylation which is produced by Streptomyces lysosuperficus (Takatsuki et al., 1971). Its mode of action has been found to be the blockage of the synthesis of N-acetylglucosaminyl-pyrophosphoryl-polyisoprenol compounds, later stages in the synthesis of saccharide-lipids being unaffected (Tkacz & Lampen, 1975; Lehle & Tanner, 1976). The structure of this anti-viral antibiotic and its site of action are shown in Fig. 5.2. Unlike sugar analogues or the amino sugars, tunicamycin does not interfere with either sugar or nucleotide metabolism. Some cytotoxic effects are evident however. Impairment of protein, DNA and RNA synthesis, and induction of RNA and DNA degradation have been shown to occur in its presence (Struck & Lennarz, 1980). The mechanism for this cytotoxicity is unknown, although it is likely that it is a secondary effect reflecting the involvement of glycoproteins in a variety of cellular processes. Numerous studies have established that tunicamycin specifically and selectively inhibits protein N-glycosylation (see Struck & Lennarz, 1980; Schwartz & Datema, 1982). It is therefore the most useful inhibitor of glycosylation for investigations on the role of N-linked oligosaccharides.

4. Other antibiotics Several other antibiotics have been found to affect glycosylation, although none of these have been as well-characterized as tunicamycin. A recent compilation of these various antibiotics may be found in Schwartz & Datema (1982).

5.1.4 Use of tunicamycin to produce aglycosyl immunoglobulins

The effect of tunicamycin on the secretion of various classes of immunoglobulin has been studied, the results suggesting that the more heavily glycosylated the immunoglobulin the more its secretion is inhibited. Thus secretion of IgG is essentially unaffected, that of IgA is much reduced, IgM secretion is almost totally inhibited and secretion of aglycosyl IgE does not occur (Hickman et al., 1977; Hickman & Kornfeld, 1978). This is probably an over-simplification however, and the extent of inhibition of secretion may be specific to the actual protein involved: IgD has been reported to be either substantially inhibited (Finkelman et al., 1981) or not inhibited at all (Sidman, 1981; Vasilov & Ploegh, 1982); and IgM from a B cell lymphoma is still secreted normally whereas that from MOPC 104E is inhibited (Sibley & Wagner, 1981). The results presented in this chapter also show that the two IgG proteins used differ in the amount of secretion of their aglycosyl forms. However, in all studies so far reported it is found that aglycosylated IgG is still secreted from cells cultured in the presence of tunicamycin (Hickman & Kornfeld, 1978; Blatt & Haimovich, 1981; Sidman, 1981), and therefore isolation of aglycosyl IgG should be possible from the culture supernatants. Because of the effectiveness and well-characterized mode of action of tunicamycin, this gives a potentially powerful means of producing carbohydrate-free IgG.

The difficulties of this approach are quite distinct from those presented by deglycosylation. The product can be made totally aglycosylated with relative ease, the problem is that it is present in only minute quantities and must be isolated from a large excess of extraneous proteins. Nevertheless, if such isolation is possible, the use of tunicamycin provides the most satisfactory form of carbohydrate-depleted protein for functional studies, and is therefore employed in these studies on the function of IgG carbohydrate.

5.1.5 Choice of IgG-producing cell line

The requisites for the line to be used are:

1. The cell line to be used must be easily grown in culture.
2. The immunoglobulin produced must be isolable from the culture supernatants. In this respect it is preferable to have an antibody which binds a simple haptenic group, facilitating purification by affinity chromatography.
3. Since the purpose of producing aglycosyl IgG is to study the effect(s) of aglycosylation on the biological functions of the immunoglobulin, the IgG produced must be of a suitable subclass. With mouse for example, this precludes the use of an IgG1 protein if Clq binding is to be studied (see section 1.6 and chapter 4).

The cell line chosen for study was K3. This is a mouse hybridoma cell line which produces an anti-DNP IgG2a protein (see Table 4.1) which can be quite easily isolated by affinity chromatography on DNP-lysine-Sepharose. This subclass performs the majority of the secondary functions of mouse IgG (see section 1.5), and is therefore useful for subsequent functional studies.

5.2 MATERIALS AND METHODS

5.2.1 Materials

All culture media were obtained from Gibco. Tissue culture vessels were from Falcon. Ficoll 400 and CNBr-activated Sepharose 4B were purchased from Pharmacia Fine Chemicals; Metrizoic acid (sodium salt, 32.8% w/v aqueous solution) and rabbit anti-bovine IgG (whole molecule) were from Sigma Chemical Co. Erythrocin B was from Difco. All radiochemicals were obtained from Amersham International. Initial experiments used tunicamycin which was a generous gift from Dr M. Potter, NIH, Bethesda, USA. The large scale culture used tunicamycin which was obtained from Sigma Chemical Co. Dinitrophenyl compounds were from BDH.

5.2.2 Preparation of affinity columns

All affinity columns were made by coupling the appropriate ligand to CNBr-activated Sepharose 4B, using the method suggested by the manufacturer.

DNP-lysine-Sepharose 100 mg of DNP-lysine was coupled per 50 ml of Sepharose. Any remaining active groups were reacted with 1 M ethanolamine.

Mouse IgG (K3) -Sepharose 10 mg of isolated K3 IgG (see section 4.2.2) was coupled to 4 ml of Sepharose as above.

Anti-bovine IgG (anti-mouse IgG activity removed) -Sepharose 1 vial of anti-bovine IgG (Sigma lot 81F-8845) was passed down the above mouse IgG -Sepharose column to remove any anti-mouse IgG cross-reactivity. The protein which passed through the column was then

coupled to 8 ml of activated Sepharose as above.

5.2.3 Cell culture media

Cells were cultured in RPMI 1640 / 10% heat-inactivated foetal calf serum (FCS) / Penicillin (100 units/ml) / Streptomycin (100 μ g/ml) / 2-mercaptoethanol (25 μ M) / glutamine (2 mM [fresh]), which is henceforth referred to as RPMI 1640 / FCS.

5.2.4 Isolation of hybridoma cells

The hybridoma lines were grown as an ascitic tumour in pristane injected Balb/c x CBA F₁ mice as described in section 4.2.2. Approximately two weeks after injection of $\sim 2 \times 10^6$ cells per mouse the ascitic fluid was removed aseptically as follows. Mice were killed by cervical dislocation, and the ventral area was swabbed with Betadine (a microbicidal solution for preparation of operative sites) followed by 70% ethanol. An incision was made down the mid-line, and the skin dissected back to reveal the peritoneum. Using a second sterile pair of scissors and forceps, a small incision was made just beneath the sternum, and the ascites was aspirated into a syringe. The fluid was added to a sterile citrate solution to prevent coagulation. These operations were carried out as rapidly as possible to prevent risk of microbial contamination. Subsequent manipulations were performed in a laminar flow cabinet (Microflow). Cells were pelleted by centrifugation at $\sim 170 \times g$ for 10 minutes, and resuspended in BSS[-Ca,-Mg] / 10% FCS / Penicillin (100 units/ml) / Streptomycin (100 μ g/ml). Viable hybridoma cells were separated from red cells and dead cells on a discontinuous Ficoll / Metrizoate density gradient (Davidson & Parish, 1975). The

hybridoma cells were recovered from the interface, diluted by addition of RPMI 1640 / FCS and pelleted at $\sim 200 \times g$ for 10 minutes. The supernatant was removed, and the cells washed by two further cycles of resuspension in RPMI 1640 / FCS followed by pelleting at $\sim 170 \times g$ for 10 minutes and removing the supernatant. The cells in the final suspension were counted in a haemocytometer and viability estimated by dye-exclusion using erythrosine B (Phillips, 1973) to be $> 96\%$. The yield of cells was $0.5-1 \times 10^8$ per mouse.

5.2.5 Monitoring the effect of tunicamycin on IgG production

The cells (2×10^6 cells ml^{-1} , 0.5 ml) with varying amounts of tunicamycin (added from a stock solution in 5 mM NaOH / 165 mM NaCl) were incubated for 3.5 hours at 37°C / 5% CO_2 in Falcon 3033 tissue culture tubes. $2 \mu\text{Ci/ml}$ of L-[4,5- ^3H]-leucine (specific activity 197 Ci/mmol) and $1 \mu\text{Ci/ml}$ of D-[1- ^{14}C]-glucosamine hydrochloride (specific activity 57.9 mCi/mmol) were then added and incubation continued for 36 hours. After centrifugation the supernatants were removed and labelled IgG isolated as below.

5.2.6 Isolation of labelled IgG from culture supernants

Labelled IgG was isolated on columns of DNP-lysine-Sepharose. After washing with PBS the IgG was eluted with 50 mM DNP-glycine pH 7.2, which was subsequently removed on a Dowex 1X8-400 column equilibrated in PBS.

5.2.7 SDS-polyacrylamide gel electrophoresis

IgG samples were run on discontinuous SDS-PAGE (Laemmli, 1970). When required, reduction of samples was with 2-mercaptoethanol. IAA was added to non-reduced samples to prevent disulphide interchange (Victoria *et al.*, 1977) and the samples were denatured in sample buffer at 100°C for 2 minutes before loading. Gels containing dual-labelled samples were sliced into 2 mm sections and the radioactivity solubilized into 0.3 mls of Protosol (New England Nuclear) : water (9:1) overnight before counting in toluene based scintillant. Fluorography was performed using EN³HANCE (New England Nuclear) and Kodak X-OMAT AR film as directed by the manufacturers. Proteins were visualized by staining with Coomassie blue (0.125%) in 7.5% acetic acid / 5% methanol and destaining by diffusion into 7.5% acetic acid / 5% methanol.

5.2.8 Biosynthetic radiolabelling at high specific activity

Since culture media contain high concentrations of unlabelled amino acid, labelling as in section 5.2.5 produces quite small incorporation of the radiolabel. This is too little to use as a tracer or to allow visualization of tritiated IgG after fluorography and so the following modification needs to be employed. RPMI 1640 medium was prepared deficient in the amino acid to be incorporated by use of the RPMI 1640 select-amine kit (Gibco). Since foetal calf serum also contains substantial amounts of free amino acids (Patterson & Maxwell, 1973), these were removed by dialysis against Dulbeccos PBS (+Ca,+Mg). These amino acid deficient components were then used instead of the normal solutions when making up the RPMI 1640 / FCS media. Cells were incubated in the modified medium ± tunicamycin for 4 hours at 37°C / 5%

CO₂. They were then washed and resuspended in fresh modified medium followed by the addition of radiolabelled amino acid (10 μCi/ml) and further incubated for 30 hours. Labelled IgG was then isolated from the supernatants as previously described.

5.2.9 Large scale production of aglycosyl IgG

Viable cells were isolated as described in section 5.2.4 and cultured in Falcon 3028 tissue culture flasks. The cells were seeded at a density of $2 \times 10^5 \text{ ml}^{-1}$ with 150 ml per flask, and subcultured every ~ 3 days as necessary by allowing them to settle, removing the spent medium and replacing it with fresh. Excess cells from cultures at ~ $8-10 \times 10^5 \text{ ml}^{-1}$ were used for aglycosyl IgG production. Periodically over the experiment radiolabelled IgG was produced (section 5.2.8) and analysed by SDS-PAGE (see for example Fig. 5.6) to ensure that no loss of secretion had occurred, that the same protein was being produced, and that binding activity remained (as a DNP-lysine-Sepharose affinity column step is employed in the purification).

When sufficient cells were available from the above cultures, these were suspended at $2 \times 10^6 \text{ ml}^{-1}$ in medium containing tunicamycin. The cells were incubated in this medium at 37°C / 5% CO₂ for 4 hours, the time required for all remaining glycosyl IgG to be secreted (Hickman & Kornfeld, 1978). After this time the cells were centrifuged at 160 x g for 10 minutes and the supernatants removed. The cells were then washed twice with fresh tunicamycin-containing medium, and resuspended at $2 \times 10^6 \text{ ml}^{-1}$. They were then cultured at 37°C for 3 days (see section 5.3.2) before removing the supernatant and storing at -20°C until required for purification as described in section 5.3.3.

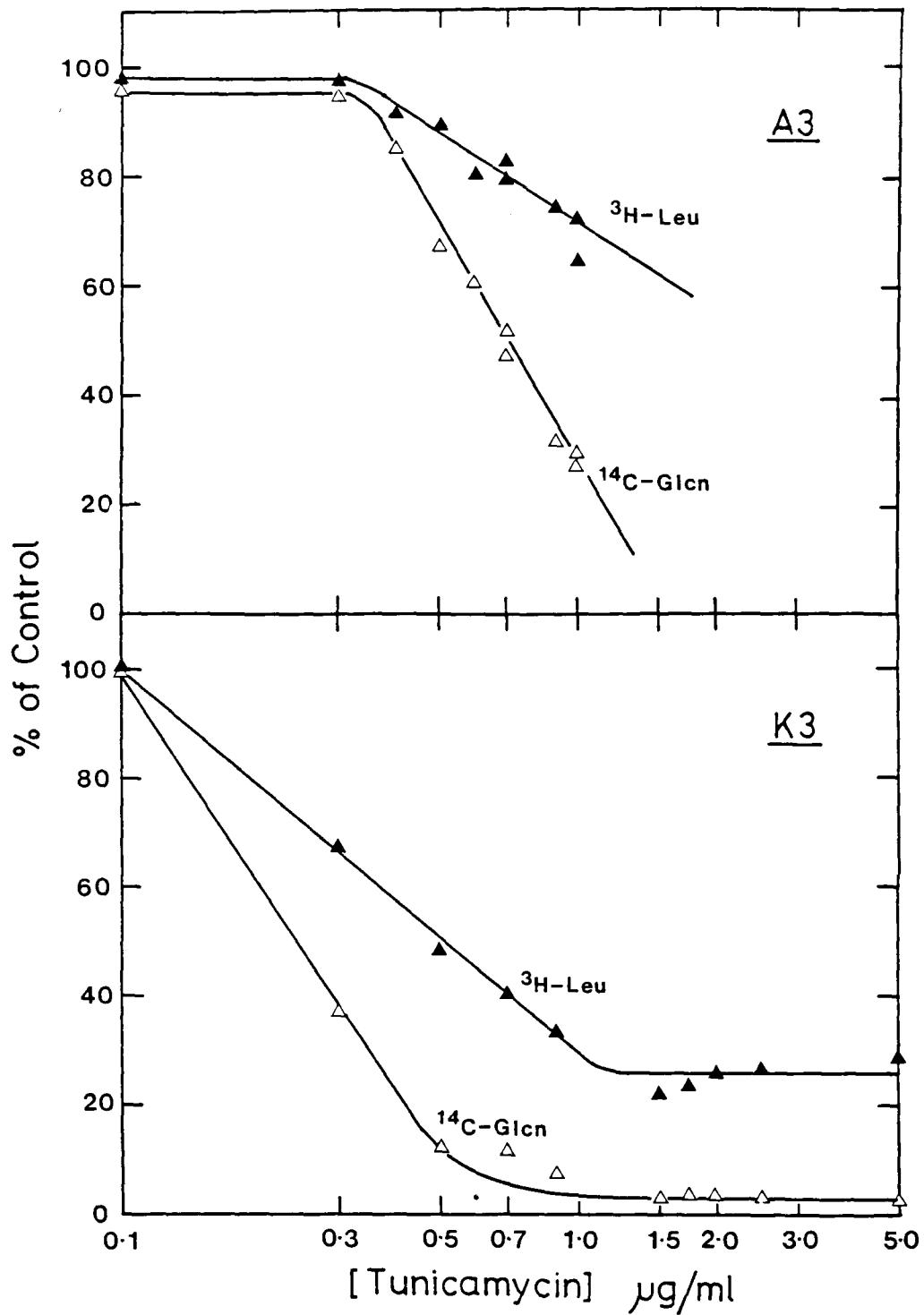


Figure 5.3 The effect of tunicamycin on the incorporation of [^3H]-leucine and [^{14}C]-glucosamine into IgG isolated from culture supernatants using the K3 and A3 cell lines.

5.3 RESULTS AND DISCUSSION

5.3.1 Effect of tunicamycin on IgG production

The effect of tunicamycin on the production of IgG by hybridoma cells in culture may be followed by the incorporation of radiolabelled amino acid. The incorporation of radiolabelled monosaccharide allows the glycosylation of the protein to be monitored. Both effects may be observed simultaneously by dual labelling. Fig. 5.3 shows the effect of increasing tunicamycin concentration on the incorporation of [³H]-leucine and [¹⁴C]-glucosamine into IgG produced by the K3 and A3 cell lines (details of these lines are given in Table 4.2). These cell lines demonstrate slightly different dose-response curves, however the overall effect is the same for each. As expected, tunicamycin inhibits the glycosylation of the immunoglobulin in a dose-dependent manner, with complete inhibition occurring at > ~ 1.5 µg/ml tunicamycin. Secretion of protein is also inhibited to some extent, but not completely. These results are in accordance with previous findings on the effect of tunicamycin production by myeloma cells, where it is found that in contrast to many other types of immunoglobulin, aglycosylated IgG is still secreted from the cells (see section 5.1.4). The inhibition of IgG secretion by the K3 cell line may seem relatively high, however this is misleading as although there is a large percentage decrease, the amount of totally aglycosyl product is actually the same as in the A3 line, the initial secretion having been higher. It is interesting to note that when the protein is completely aglycosylated no further inhibition of secretion occurs. This suggests that it is only aglycosylation which is responsible for the decrease, this being either a direct effect on the IgG or a secondary effect due to the aglycosylation

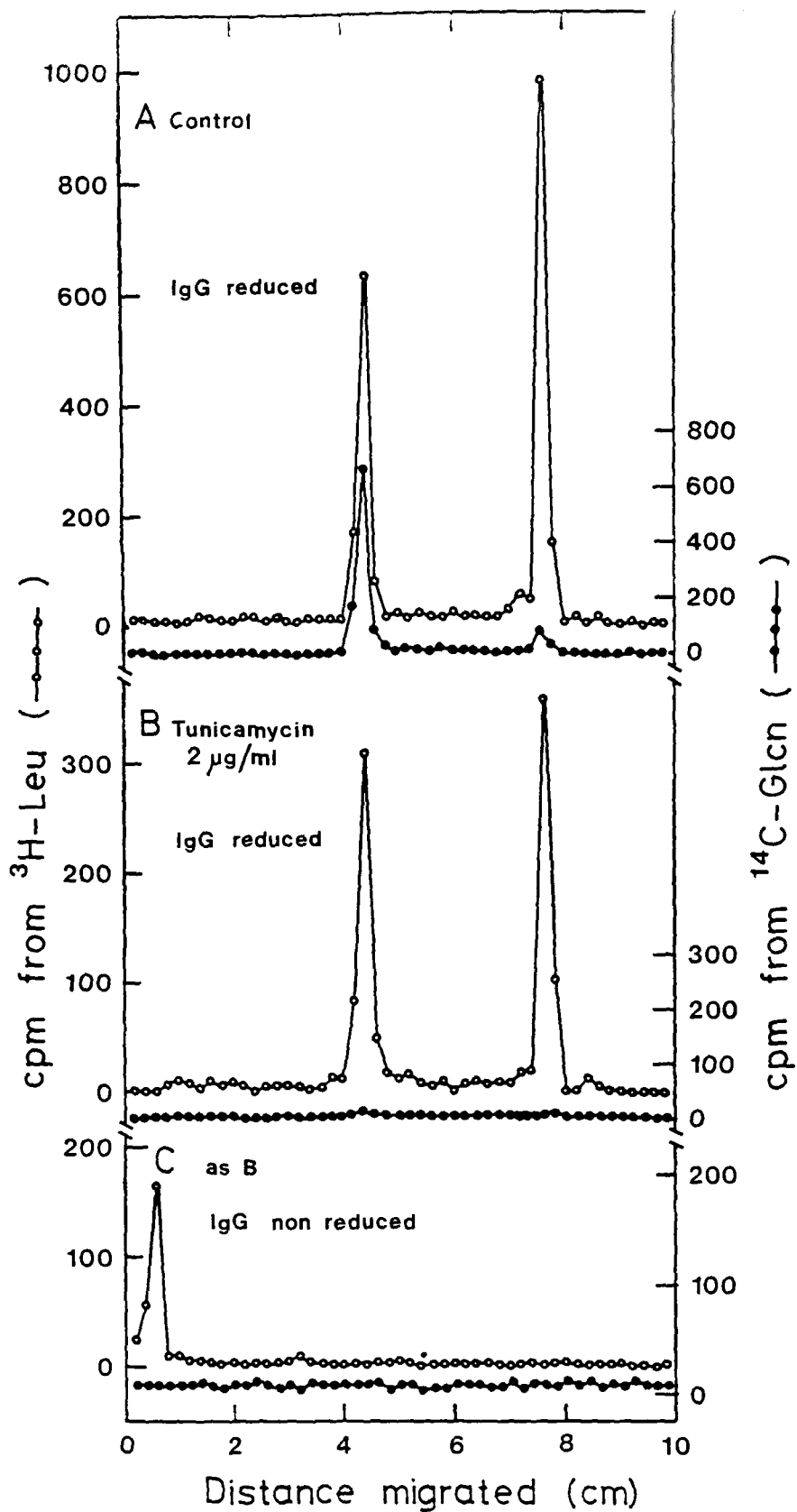


Figure 5.4 SDS-PAGE (9% acrylamide) of IgG synthesized by the K3 cell line cultured in medium containing [³H]-leucine and [¹⁴C]-glucosamine. (A) in the absence of tunicamycin, (B,C) in the presence of 2 µg/ml tunicamycin. In (A) and (B) the samples were reduced with 2-mercaptoethanol, (C) is unreduced.

of some other protein(s). This result also indicates that a slight excess of tunicamycin (to ensure complete aglycosylation) may be used without risking further inhibition of IgG secretion. For subsequent experiments with the K3 cell line a concentration of 2 $\mu\text{g}/\text{ml}$ tunicamycin was therefore used (for this batch of tunicamycin).

The SDS-PAGE profiles of this dual-labelled IgG from K3 cells, produced in the absence or presence of 2 $\mu\text{g}/\text{ml}$ tunicamycin and isolated on DNP-lysine-Sepharose columns are shown in Fig. 5.4. The results are as follows:

1. The isolation procedure yields radio-chemically pure IgG.
2. Glycosylation (at least with amino sugars) occurs only on the H chain of this protein.
3. The presence of 2 $\mu\text{g}/\text{ml}$ tunicamycin completely blocks this glycosylation. It should be noted that since tunicamycin only prevents N-linked glycosylation, and since the glucosamine would be expected to be incorporated into GlcNAc, GalNAc and sialic acid residues, this shows that O-linked carbohydrate is probably not present on this protein.
4. The assembly of the IgG into a disulphide-linked H_2L_2 unit still occurs normally in the absence of glycosylation.

These results establish that it is possible to produce IgG from the K3 cell line which is aglycosylated and assembled as a complete H_2L_2 unit by culturing isolated cells in medium containing tunicamycin. It is also possible to isolate the resultant protein by affinity chromatography using a support derivatized by its natural DNP antigen (control experiments (not shown) where the IgG was isolated by use of a double antibody system [rabbit anti- mouse IgG + goat anti- rabbit IgG]

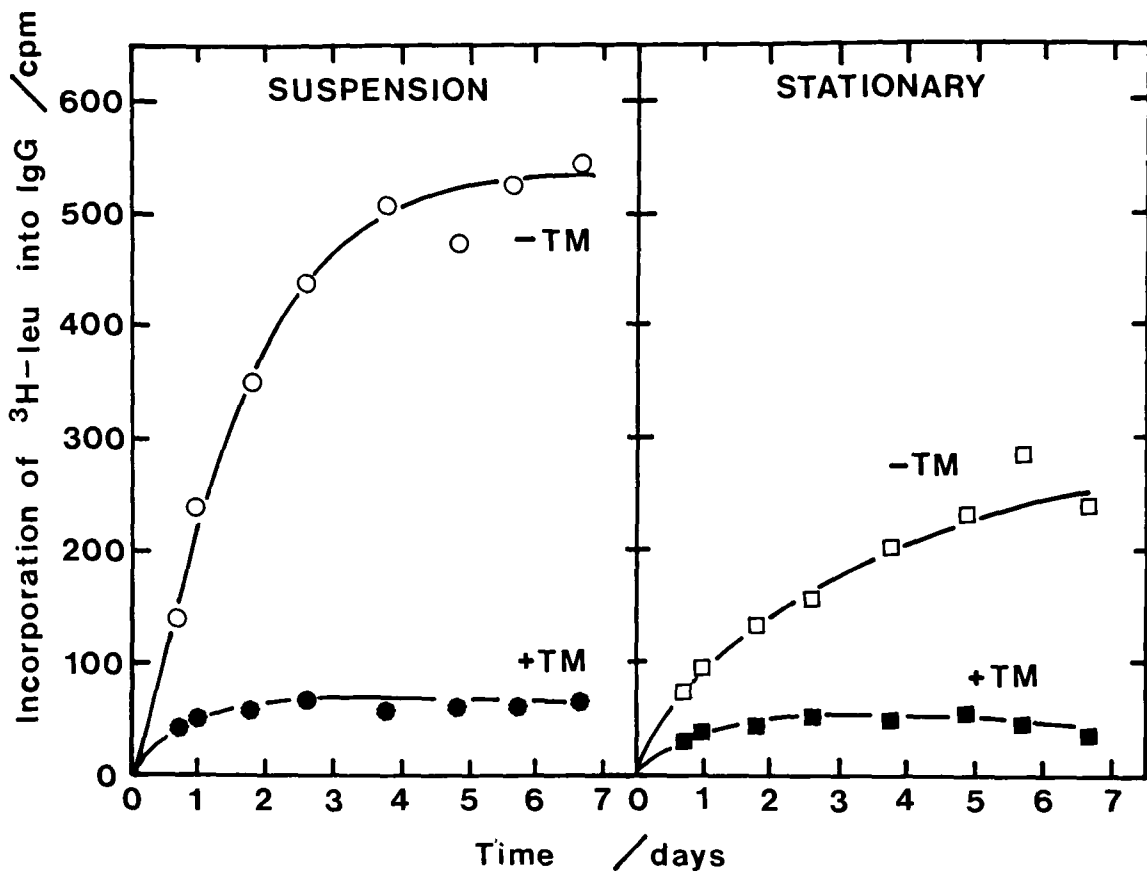


Figure 5.5 Time course of IgG production (incorporation of [³H]-leu per 50 μ l, [³H]-leu in the medium = 2.6 μ Ci/ml) by K3 cells cultured in the presence and absence of tunicamycin (TM), and in suspension or stationary cultures. .

had shown that all the aglycosyl IgG bound to the DNP column).

5.3.2 Production of aglycosyl IgG in culture

As a pre-requisite to the large scale production of aglycosyl IgG it is necessary to determine the optimal time / conditions for harvesting of the culture supernatants. Therefore the amount of IgG produced was monitored from cells grown in static and suspension cultures ± tunicamycin over a seven day period. The results are shown in Fig. 5.5. In the absence of tunicamycin, twice as much IgG is secreted by cells in suspension culture as in static culture. With tunicamycin there is a drastic reduction in the IgG produced, with no more secretion occurring after three days in culture. Suspension culture in the presence of tunicamycin gives little benefit in terms of yield and so, since such cultures are more difficult to maintain, the conditions for further large-scale production of aglycosyl IgG were decided to be stationary cultures with harvesting of the supernatant after three days.

The large scale production of aglycosyl IgG was as follows. Hybridoma cells were isolated from the ascites of tumour-bearing mice as described in section 5.2.4, and cultured in RPMI 1640 / FCS at 37°C in a humidified incubator (Gallenkamp) with a 5% CO₂ atmosphere. Viability was maintained at > 95%. Cells were harvested and grown in the above medium containing tunicamycin (see section 5.2.9) to produce a total of 2.5 l of aglycosyl IgG-containing supernatant. The concentration of mouse IgG in this supernatant was estimated to be ~ 1-2 µg/ml by single radial immunodiffusion (Mancini et al., 1965). Glycosylated IgG was also produced as a control from culture of cells in the absence of

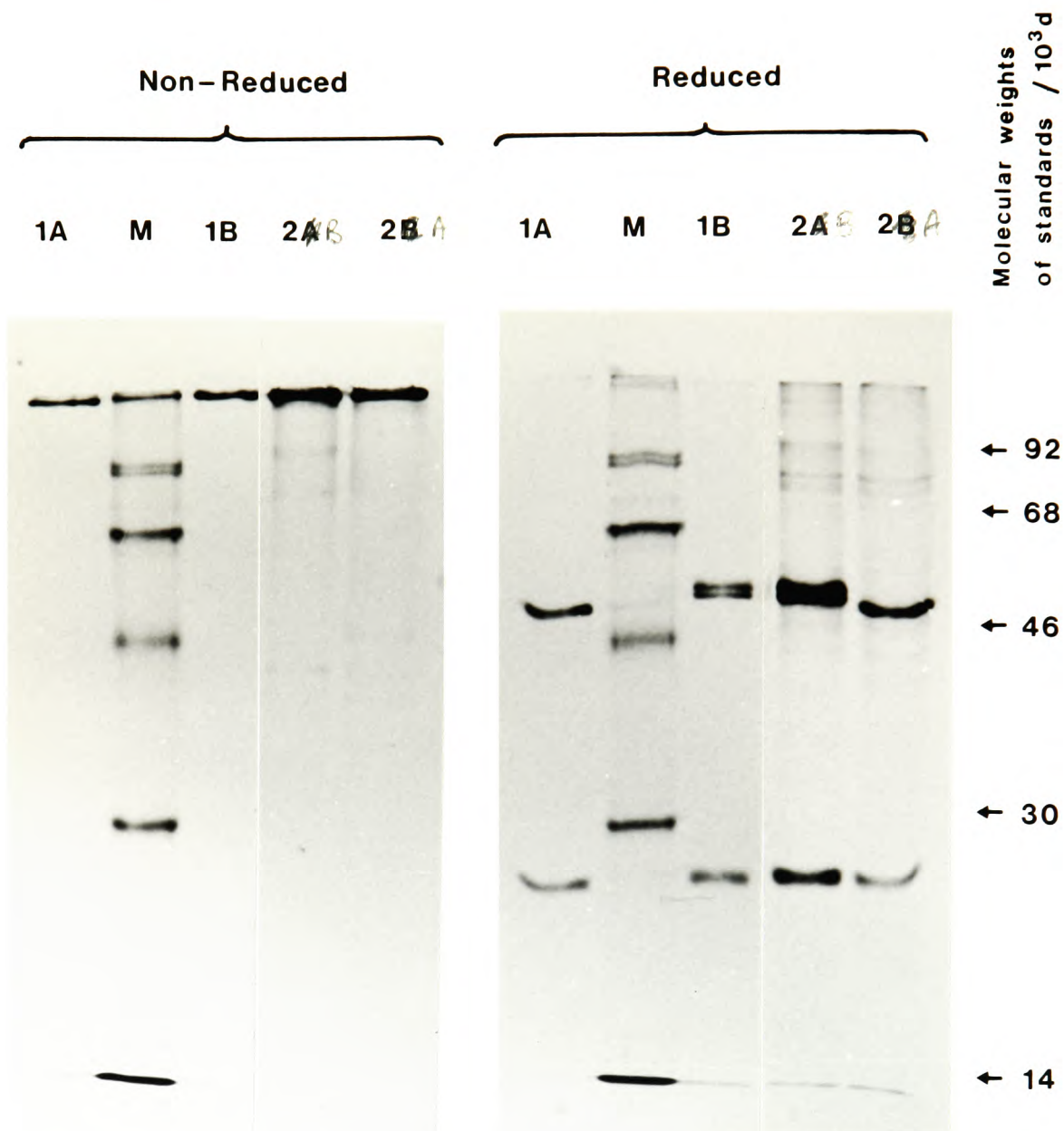


Figure 5.6 Fluorograph of a 10% acrylamide SDS-PAGE gel of reduced and non-reduced biosynthetically [³H-leucine] (1), and [³⁵S-methionine] (2) labelled IgG produced (A) in the presence, and (B) in the absence of tunicamycin. 26,000 dpm were applied per track, and the film was exposed at -70°C for 3 weeks. Also shown are the positions of [¹⁴C]-labelled molecular weight standards (M).

tunicamycin.

5.3.3 Purification of aglycosyl IgG from culture supernatants

The following protocol was followed for both the glycosyl and aglycosyl culture supernatants. All purification steps were performed at 4°C. The supernatants were concentrated tenfold by ultrafiltration on an Amicon PM30 membrane, dialysed versus PBS / EDTA, and any precipitated material removed by centrifugation. The supernatant was then applied to a DNP-lysine-Sepharose column (5 x 1.4 cm) in PBS / EDTA, and unbound material washed through with the same buffer. Also applied to the column was biosynthetically labelled [³H-leucine]-IgG-containing supernatant, prepared as described in section 5.2.8. Fluorography of SDS-PAGE performed on IgG isolated from this supernatant is shown in Fig. 5.6. This shows the IgG to be radiochemically pure, and so it may be used as a tracer. Bound protein was eluted with 50 mM DNP-glycine pH 7.2. After concentration this protein was applied to a Sephacryl S-200 column (140 x 1.6 cm) in PBS / EDTA to further purify the product and remove excess DNP-glycine. Monitoring the absorbance of the eluate at 280 nm revealed two peaks, one eluting with the void volume, and the second at a position corresponding to that of the radiolabelled K3 IgG i.e. at ~ 150 kDaltons. The radioactivity profile showed no evidence of lower molecular weight degradation or misassembly products. The SDS-PAGE profiles of the fractions from these various steps are shown in Figs. 5.7 and 5.8. The following observations may be made from the unreduced samples (Fig. 5.7):

1. The DNP-lysine-Sepharose affinity column step removes the vast majority of the contaminating bovine proteins (present from the foetal calf serum), but some non-IgG impurities remain.

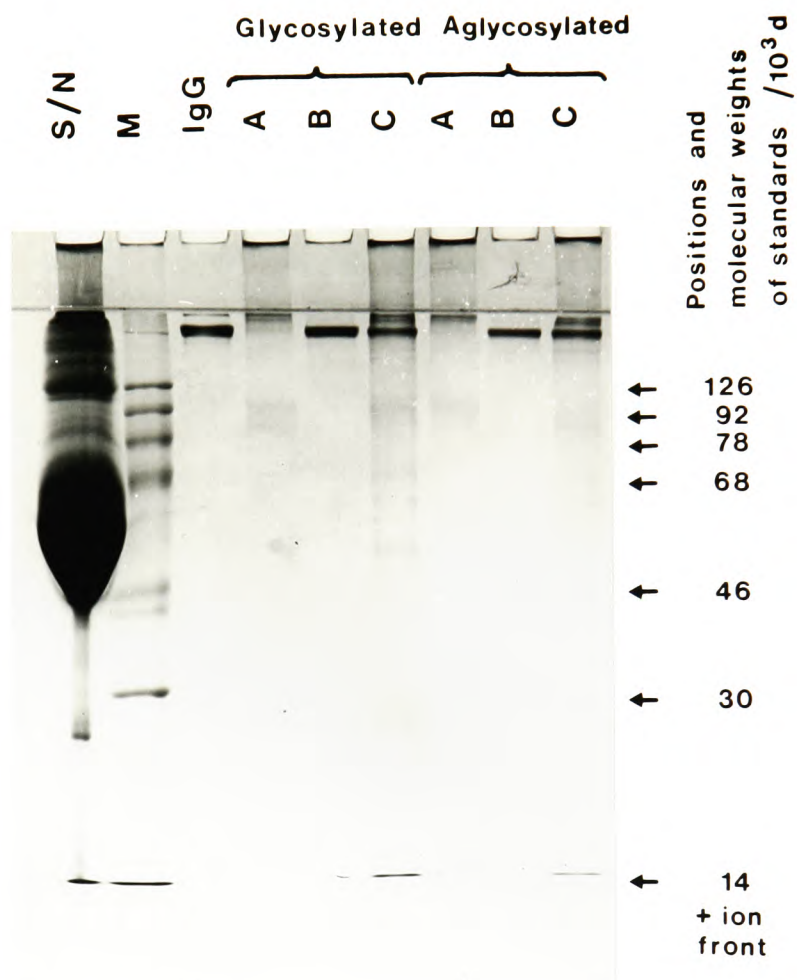


Figure 5.7 10% SDS-PAGE of unreduced:

S/N The whole culture supernatant prior to application to the DNP-lysine-Sepharose column

C The fraction which bound to this column

A The high molecular weight fraction obtained when fraction (C) was passed down the S-200 gel filtration column

B The 150 kDalton fraction from the S-200 column

IgG Purified mouse IgG (K3) from ascitic fluid

M Molecular weight standards

The molecular weights of the standards are arrowed. Proteins were visualized by staining with Coomassie blue.

2. The gel filtration column allows isolation of pure 150 kDalton component(s), which correspond in position of elution to authentic K3 IgG.
3. The material eluting in the void volume does not contain any IgG, and consists of components of extremely high apparent molecular weight, many of which do not enter the 5% acrylamide stacking gel.

Examination of the reducing gel (Fig. 5.8) reveals that an additional diffuse band (marked by an asterisk) is present in the material isolated from cell culture, both in the presence and absence of tunicamycin. This band occurs at $M_r = 59$ kDaltons compared with a value of 55 kDaltons for the mouse IgG heavy chain. Fluorography of isolated biosynthetically labelled IgG secreted by these cells (Fig. 5.6) shows that this component is not produced by the hybridoma cells. A likely identity of this band is the heavy chain of bovine IgG (present from the foetal calf serum), due to a cross-reaction of a small proportion of such IgG with DNP. This was confirmed in two ways. Firstly the position of this band on SDS-PAGE coincided with the position of the H chain of an authentic bovine IgG sample (see Fig. 5.9). Secondly, a precipitin arc was formed by the 150 kDalton fractions against both anti-bovine IgG and anti-mouse IgG (results not shown). Authentic bovine and mouse (K3) IgGs only produced a precipitin arc against their respective antisera.

Removal of this bovine IgG was achieved by passage of the 150 kDalton fractions through an anti-bovine IgG -Sepharose column (any anti-mouse IgG activity in this antisera had been removed prior to coupling to the Sepharose by passage down a mouse IgG (K3) -Sepharose column). The SDS-PAGE profiles of the 150 kDalton fractions before and

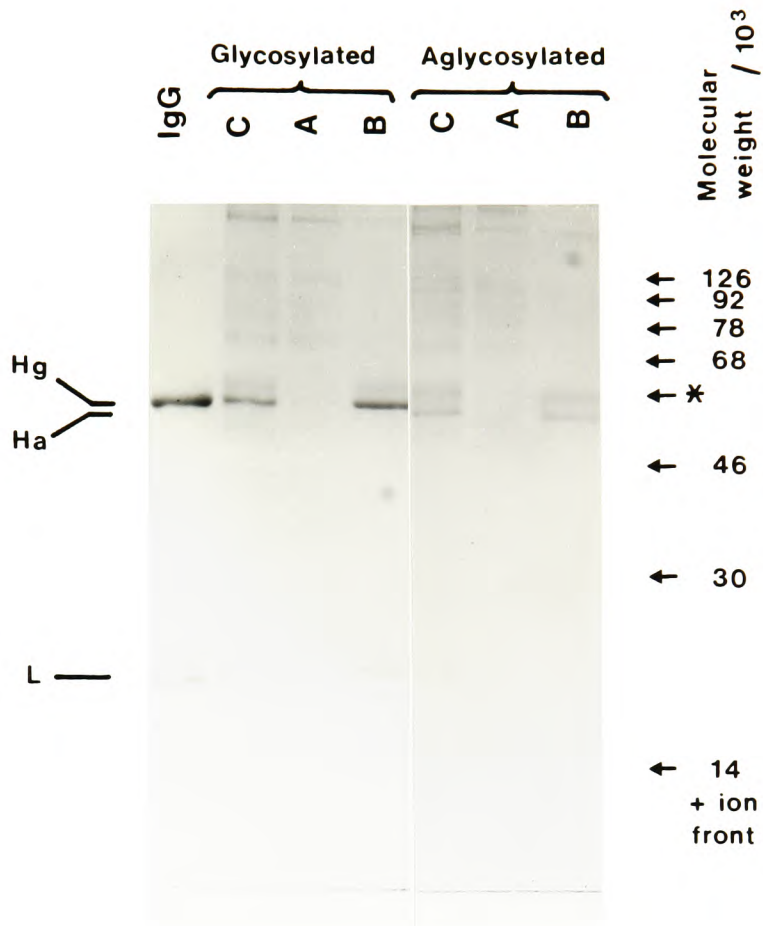


Figure 5.8 10% SDS-PAGE of the fractions described in Fig. 5.7, but with samples reduced with 2-mercaptoethanol. The gel was stained for protein using Coomassie blue. Tracks are labelled as Fig. 5.8 plus:

- Hg Position of glycosylated heavy chain
- Ha Position of aglycosylated heavy chain
- L Position of light chain
- * Additional "diffuse" band

The positions of molecular weight markers are arrowed.

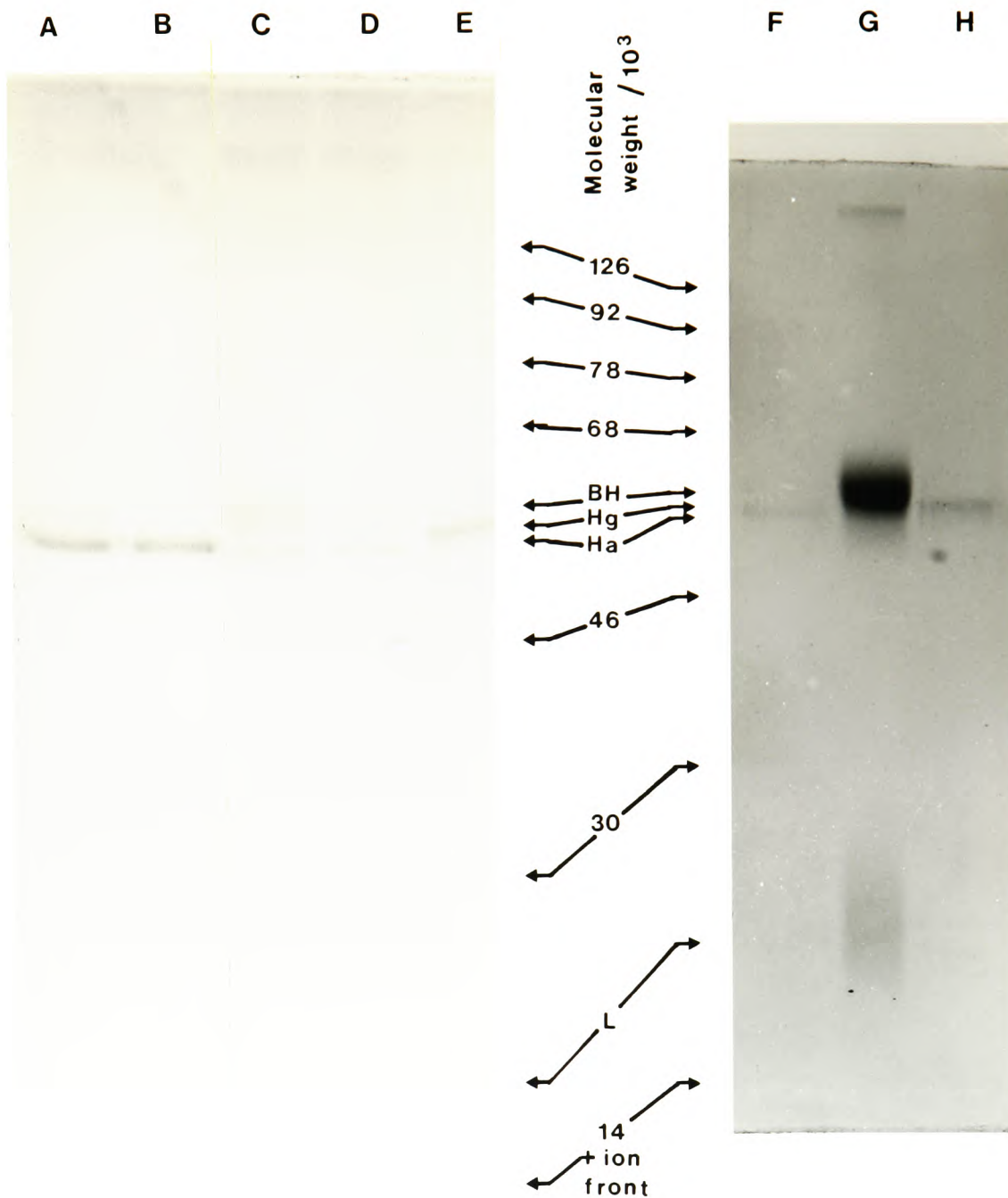


Figure 5.9 Removal of bovine IgG by passage of samples through an anti-bovine IgG -Sepharose column. Shown is SDS-PAGE (10% acrylamide, samples reduced with 2-mercaptoethanol) of:

- A Glycosyl IgG before passage through column
- B Glycosyl IgG after passage through column
- C Aglycosyl IgG before passage through column
- D Aglycosyl IgG after 1st passage through column
- E Mixture of glycosyl and aglycosyl IgG
- F Aglycosyl IgG after 2nd passage through column
- G Bovine IgG
- BH Position of bovine IgG heavy chain

The gels were stained for protein using Coomassie blue.

after the anti-bovine IgG column are shown in Fig 5.9. As is seen, the greater relative contamination of the aglycosyl IgG sample required two passages to obtain a pure product. An additional feature of these gel profiles is that a mixture of glycosyl and aglycosyl IgG shows two distinct positions for the migration of their respective H chains, with the latter having the expected lower apparent M_r for an aglycosyl polypeptide on SDS-PAGE due to the reduced SDS binding of saccharide compared with peptide portions of proteins (Schubert, 1970; Segrest et al., 1971). This difference is also seen in the fluorograph of Fig. 5.6, where the better resolution also shows that the more diffuse glycosylated band actually runs as a 'doublet'. This has also been observed for IgG synthesized by a human lymphoblastoid cell line (Owen & Kissonerghis, 1982), and must be due to glycosylation differences. Small differences in the oligosaccharide chain can affect migration in SDS gels - differing sialylation could cause these effects (Segrest et al., 1971). The other possibility of a second glycosylation site which was partially glycosylated can be discounted, as K3 protein has been shown by quantitative hydrazinolytic cleavage to contain only two N-linked chains per molecule (T. W. Rademacher, personal communication) i.e. only a single oligosaccharide per H chain.

The SDS-PAGE profiles of the final purified proteins are shown in Fig. 5.10. The different migration of the H chains is again visible, and from this it can be seen that the aglycosylated protein contains essentially no contaminating glycosyl components and that neither preparation shows the presence of any other protein bands. They are therefore suitable for use in assays to determine the properties of the aglycosyl protein. The total yield of aglycosyl IgG was ~ 1 mg.

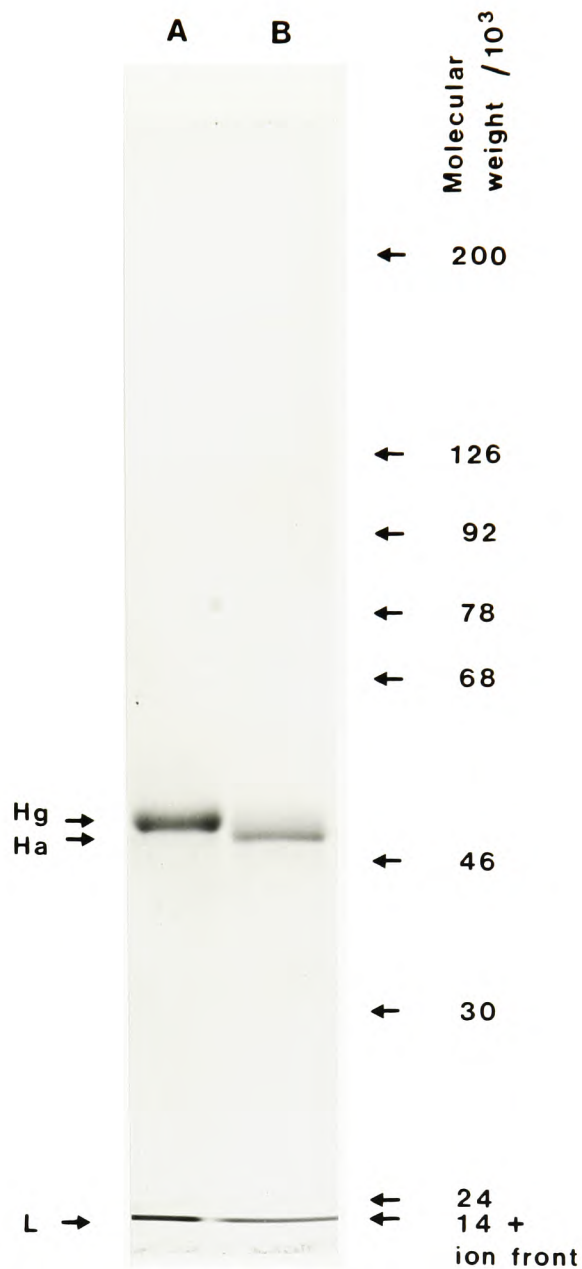


Figure 5.10 SDS-PAGE (9% acrylamide, samples reduced with 2-mercaptoethanol) of the purified (A) glycosyl, and (B) aglycosyl IgG as used for the functional studies described in chapter 6. The gel was stained for protein using Coomassie blue.

Hg Position of glycosylated IgG heavy chain

Ha Position of aglycosylated IgG heavy chain

L Position of light chain

The positions of various molecular weight standards are arrowed.

5.4 SUMMARY

The continued secretion of aglycosyl IgG from cells cultured in the presence of tunicamycin was confirmed using available hybridoma cell lines. The aglycosyl IgG was shown to be assembled as a normal, antigen binding H_2L_2 unit, which could be quantitatively isolated on DNP-lysine-Sepharose affinity columns.

Large scale culture of K3 cells in the presence of a suitable concentration of tunicamycin was performed to obtain sufficient quantities of IgG to be useable in functional assays. Isolation of protein which bound to DNP-lysine-Sepharose, followed by gel-filtration on Sephacryl S-200 gave a product which contained the synthesized mouse IgG, together with some natural anti-DNP IgG antibodies from the foetal calf serum of the culture medium. This bovine IgG was removed by an anti-bovine IgG (anti-mouse IgG cross-reactivity removed) -Sepharose affinity column step. The remaining protein was pure mouse IgG which was essentially totally aglycosylated, as judged by its slightly greater mobility on SDS-PAGE compared with normal glycosylated IgG (produced from culture in the absence of tunicamycin, and isolated in the same manner as the aglycosyl IgG). The functional properties of this aglycosyl IgG are investigated in chapter 6.

CHAPTER 6

Comparison of the Functional Properties of Glycosyl and Aglycosyl IgG

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6.1 INTRODUCTION

Before examining the possible role of the carbohydrate in IgG, it is worthwhile to briefly review the evidence for the significance of glycosylation in other protein systems. As well as being useful in possibly suggesting similar functions in IgG this allows an estimation of the overall importance of oligosaccharide attachment for glycoprotein function.

6.1.1 Recognition of glycoprotein carbohydrate by receptors

In mammalian systems it is found that desialylated serum glycoproteins have vastly reduced survival times in the circulation when compared with the native form of the same protein. This clearance has been found to be mediated by a galactose-specific receptor located on hepatocytes which is responsible for endocytosis of such proteins (see reviews by Neufeld & Ashwell [1980], and Ashwell & Harford [1982]). Its specificity is such that binding of asialo oligosaccharide chains follows the series tetraantennary > triantennary >> biantennary >> monoantennary \approx galactose (Lee et al., 1983). Other receptors which also mediate clearance are the mannose / N-acetylglucosamine receptor (Stahl & Schlessinger, 1980), and the α 1-3 fucose receptor (Prieels et al., 1980). As was discussed in section 1.9.1 the role of the carbohydrate on IgG in determining catabolic rate is controversial, and in any case does not explain the relatively long half-life of IgG in the circulation (Table 1.2).

Another carbohydrate receptor system which is well-characterized is the lysosomal phosphomannose receptor. It has been established that

lysosomal enzymes bear a recognition marker for their transfer into the lysosomes. This marker appears to be mannose-6-phosphate (Kaplan et al., 1977; Fischer et al., 1980), or its biosynthetic precursor α -N-acetylglucosamine-1-phospho-6-mannose (Haslik et al., 1980; Goldberg & Kornfeld, 1981), as part of the protein oligosaccharide. The importance of the phosphorylation is shown by the existence of the genetic disorder I-cell disease in which storage and processing of certain lysosomal enzymes is impaired. It has been found that the defect in I-cell disease is the inability to phosphorylate these enzymes (Haslik & Neufeld, 1980).

6.1.2 Effect of carbohydrate removal on glycoprotein function

Table 6.1 lists some of the extensive literature on the role of carbohydrate in various glycoproteins (although see also the review by Olden et al., 1982). In general it is noted that:

1. There is usually no effect on the enzymic activity of glycoenzymes.
2. Lack of carbohydrate can lead to reduced solubility.
3. Aglycosyl proteins often exhibit much increased proteolytic susceptibility.

It was originally proposed that the role of carbohydrate on glycoproteins was to allow secretion to occur (Eylar, 1966). This has subsequently been shown to be incorrect, and the determining factor found to be an N-terminal sequence of hydrophobic amino acids termed the "signal peptide" (Milstein et al., 1972; Blobel & Dobberstein, 1975a,b; Rothman & Lodish, 1977; Braell & Lodish, 1982; von Heijne, 1982). In spite of this, tunicamycin treatment often results in reduced secretion rates of various proteins. This seems however to be a result of one or

both of points (2) and (3) above rather than a direct effect on secretion (Olden et al., 1982).

When interpreting experiments which utilize tunicamycin it is extremely important to note the effect of such treatment on the sub-cellular localization of the lysosomal enzymes. Without N-glycosylation these enzymes are unable to interact with the phosphomannose receptor (see section 6.1.1). The net result is that lysosomal enzymes including proteases will be released into the medium (Olden et al., 1982). In conjunction with the usually enhanced protease susceptibility of aglycosyl proteins this may lead to much of the observed increased degradation of proteins during tunicamycin treatment. Indeed, Olden et al. (1982) have demonstrated that much of the cytotoxic effect of tunicamycin can be reversed by addition of protease inhibitors.

Nevertheless, specific effects of aglycosylation on protein function do occur. These effects may be due to two factors. Firstly the carbohydrate moiety may be required to interact directly with further systems e.g. the phosphomannose receptor. There is good evidence that this is a function of certain glycoprotein oligosaccharides, and by definition it depends on the sequence of the oligosaccharide. A second possible effect is that the addition of carbohydrate may be necessary in order to achieve a correct protein tertiary structure. Although it has been suggested that the presence of N-linked carbohydrate may assist correct folding of a glycoprotein (Chu et al., 1978; Gibson et al., 1979, 1981; Kaluza et al., 1980; Crimmins & Schlessinger, 1982) the physical evidence that addition of oligosaccharide causes gross changes in protein structure is limited. In

this respect renaturation experiments + carbohydrate (on which much of the evidence is based) are possibly not a good model, as during biosynthesis the N-linked sugar is added after a substantial proportion (although not all) of the protein has been synthesized (Kiely et al., 1976; Rothman & Lodish, 1977; Bielineska & Biome, 1978), and processing of terminal sugars occurs even later (Tartakoff & Vassalli, 1979). The only structural comparisons to date of proteins with and without oligosaccharide find no major conformational differences (Puetz, 1973; Wang & Hirs, 1977; Berman et al., 1981). This latter observation is also implied in the many instances that aglycosylation has no effect on biological function (see Table 6.1).

One group of glycoproteins which are attracting a lot of recent attention are the glycoprotein hormones. As listed in Table 6.1 several reports indicate that although glycosylation is not necessary for binding to their receptor, lack of carbohydrate, which is accounted for by lack of the terminal sialic acid and galactose residues (Moyle et al., 1975; Kalyan et al., 1982) results in a loss of biological activity. The reason for this is not presently known.

6.1.3 Role of carbohydrate in immunoglobulins

The effects of carbohydrate removal on immunoglobulins are summarized in Table 6.2. Apart from the previously noted intermittent reduction of secretion of certain immunoglobulins (other than IgG) (see section 5.1.4) the following effects of carbohydrate removal may be noted:

1. There appears to be minimal effect on quaternary assembly (also found in chapter 5). There is however a single report of incorrect

Table 6.2

The effect of carbohydrate depletion on immunoglobulins of various class

Ig	METHOD	EFFECT OF DEPLETION	REFERENCES
A	tunicamycin	Reduction of secretion, still binds hapten. No effect on expression at cell surface.	Hickman <u>et al.</u> , 1977; Hickman & Kornfeld, 1978; Hickman & Wong-Yip, 1979
	tunicamycin	No inhibition of secretion	Williamson <u>et al.</u> , 1980
D	tunicamycin	Substantial loss of secretion	Finkelman <u>et al.</u> 1981
	tunicamycin	No effect on secretion	Sidman, 1981; Vasilov & Ploegh, 1982
E	tunicamycin	Total inhibition of secretion	Hickman <u>et al.</u> , 1977
	tunicamycin	No effect on receptor binding	Kulczycki & Vallina, 1981
G	mixed glycosidases	No effect on placental transport	Miller, 1971
	mixed glycosidases	No effect on antigen binding, variable effect on complement fixing and opsonic activity	Williams <u>et al.</u> , 1973
	2-deoxy-D-glucose	Sub-cellular localization affected	Melchers, 1973
	mutant cell line	No effect on secretion	Weitzman & Scharff, 1976
	mixed glycosidases	Complement fixation, rosette formation and ADCC reduced. Antigen binding unaffected	Koide <u>et al.</u> , 1977
	endoglycosidase	Clq binding abolished, antigen binding unaffected	Winkelhake <u>et al.</u> , 1980
	tunicamycin	No significant effect on secretion. Assembly not affected	Hickman & Kornfeld, 1978; Blatt & Haimovich, 1981; Sidman, 1981

...continued

Ig	METHOD	EFFECT OF DEPLETION	REFERENCES
M	endoglycosidase H	Decreased solubility, no effect on complement	Tarentino <u>et al.</u> 1974
	tunicamycin	Substantial loss of secretion No affect on assembly	Hickman & Kornfeld, 1978
	tunicamycin	Substantial loss of secretion	Blatt & Haimovich, 1981; Sidman, 1981
	tunicamycin, deoxyglucose	Chain assembly, polymerization, and secretion (rate reduced) still occur	Tartakoff & Vassalli, 1979
	tunicamycin	No effect on secretion or cell surface expression; increased proteolysis	Sibley & Wagner, 1981
	tunicamycin	No effect on secretion	Vasilov & Ploegh, 1982
	mutant cell lines, <u>+</u> tunicamycin	Generally no increase in degradation, however one clone has reduced degradation rate when aglycosylated	Sidman <u>et al.</u> , 1981
	tunicamycin	Increased degradation of membrane IgM	Dulis <u>et al.</u> , 1982
	tunicamycin	Cell surface expression of membrane IgM prevented. No increase in degradation rate	Yuan, 1982
	tunicamycin	Non-glycosylated J-chain is still incorporated	Williamson <u>et al.</u> , 1980
	tunicamycin	Incorrect chain assembly	Kubo & Pelanne, 1983

assembly of aglycosyl IgM (Kubo & Pelanne, 1983), although the evidence on which this is based (non appearance of a band on SDS-PAGE) is tenuous.

2. The more glycosylated molecules may lose solubility on removal of carbohydrate (possibly accounting for their reduced secretion).
3. In common with observations on many proteins (see section 6.1.2) IgM seems to become much more susceptible to proteolysis. No evidence is available for the other immunoglobulin classes.
4. A few reports suggest that while deglycosylated IgG retains its antigen-binding activity, it has reduced ability to fix complement and bind to cellular receptors.

Since the purpose of this chapter is to examine the effect of aglycosylation on the secondary functions of IgG, point 4 is extremely relevant and therefore the available data are examined more closely. Williams et al. (1973) found that upon removal of 60% of the carbohydrate from rabbit IgG by glycosidase digestion three out of the four preparations tested exhibited no change in complement-fixing, opsonic or agglutinating activities; however one preparation completely lost both complement-fixing and opsonic activity. Koide et al. (1977), again using glycosidase digestion, claimed 84% removal of carbohydrate from rabbit anti-sheep red blood cell IgG, and obtained the following results: no effect was found on haemagglutination; significant effects were noted on rosette formation, antibody-dependent cell-mediated cytotoxicity (ADCC), and complement fixation.

At this point it must be noted that there is a great difference between interpreting effects on a system which uses monomeric IgG, and one in which the IgG is required to be aggregated in some form. This is

due to the effect that a small proportion of residual glycosylated IgG (as would be expected in a glycosidase treated preparation) would have on the overall activity of the preparation. This is best illustrated by considering the activity of a preparation of IgG which is 90% modified and inactive, contaminated by 10% residual unmodified and active IgG, in assays dependent on monomeric and aggregated IgG. In an assay which depended on monomeric IgG the above preparation as a whole would only exhibit a ten-fold loss in activity i.e. the net activity would be equal to the contamination level. In contrast if the assay was dependent on the IgG being grouped by aggregation (as in the case of Clq or C1 binding) then the effect of the same level of contamination is much less. This is because the chance of the contaminating normal IgG molecules being grouped together and so able to bind Clq is small. Consequently in this type of assay the net activity of the preparation would be substantially less than the contaminant level.

In the light of the above considerations the results of Koide et al. (1977) can be interpreted as follows. Their dose-response curve for ADCC versus concentration of monomeric IgG shows a factor of ~ 6 difference between the glycosyl and deglycosyl IgG. Since this could be accounted for by a contamination level of 17% by normal, undigested IgG in their deglycosyl preparation (not an unreasonable estimation of the purity) it is possible that the actual deglycosylated IgG molecules in their preparation displayed no activity, i.e. the effect seen is not significantly higher than the contamination level. A similar level of reduction of activity (five-fold) is found with complement-dependent haemolysis. Yet by the arguments above this would require either a level of contamination by normal IgG which was much higher than that required to explain the ADCC results, or mean that the deglycosyl IgG

was active, and itself gave rise to this haemolysis, albeit more slowly. The level of contamination which would be required if the deglycosyl IgG were totally inactive can actually be estimated directly. Minami & Utsumi (1981) report an elegant study where haemolysis was monitored with varying proportion of a complement-fixing antibody (guinea pig IgG2) and a non-complement-fixing antibody (guinea pig IgG1) bound to trinitrophenylated sheep red blood cells. As expected from the necessary grouping of the IgG2 needed to allow C1 fixation, the dependence of relative haemolysis on proportion of IgG2 is distinctly non-linear. Since this is directly comparable with the situation described above, if deglycosylated IgG was totally inactive then from the data of Minami & Utsumi (1981) the amount of contaminating normal IgG required to account for the residual complement-fixing activity would be 55%. This is clearly far too high (and inconsistent with the maximal level of 17% estimated previously), and therefore the deglycosylated IgG must still retain some complement-fixing ability.

6.1.4 Interaction of IgG with protein A

Immobilized Staphylococcal protein A (SpA) has been widely used in the affinity purification of IgG. More recently it has been found that the conditions for elution of IgG from SpA-Sepharose differ for different IgG subclasses. This has been used in the purification of IgG subclasses of many species (Ey et al., 1978; Duhamel et al., 1979; Delacroix & Vaerman, 1979; Coe et al., 1981; Ricardo et al., 1981; Nilsson et al., 1982). Perhaps the best example is with mouse IgG (Ey et al., 1978), where each of the main subclasses (1, 2a and 2b) may be separated from each other by their position of elution from SpA-Sepharose using a series of buffers of descending pH. Since no

other mouse Ig class binds, this affords a rapid and effective purification protocol which would be extremely difficult by more conventional techniques. By implication therefore, the position of elution from an SpA-Sepharose column by a descending pH gradient reflects the differing structure / sequence around the SpA binding site. This is well illustrated in the case of human IgG3 which is not usually considered to bind protein A. The lack of binding is explained by the substitution of His-435 in the SpA binding site by an arginine residue; restoring the histidine as is found in a γ 3 allotypic variant, restores binding (Recht et al., 1981).

A study of the binding of aglycosyl IgG to SpA is therefore useful for two reasons. Firstly, if binding remains it provides a further purification protocol, if required. Secondly, the binding to, and conditions of elution from SpA-Sepharose offer an indication of any structural changes around the SpA binding site. Such an approach has been used to show that the hinge-deleted human IgG1(Dob) binds SpA normally (Klein et al., 1981), reflecting the normal Fc structure found in X-ray crystallographic studies on this protein (Silverton et al., 1977).

6.2 MATERIALS AND METHODS

6.2.1 Materials

Protein A-Sepharose was purchased from Pharmacia. [¹²⁵I]-C1 was generously provided by B. Gorick & N. R. Hughes-Jones, A.R.C., Cambridge. [¹²⁵I]-human IgG (0.015 I/IgG) iodinated by use of enzymebeads (Bio-Rad) was provided by J. Woof & D. R. Burton, University of Sheffield. Glycosyl and aglycosyl mouse IgG2a (K3) were prepared as described in chapter 5. Pepsin was obtained from Worthington.

6.2.2 Protein A -Sepharose chromatography

Radio-labelled glycosyl and aglycosyl IgG were produced as described in section 5.2.5. The labelled IgG was isolated on columns of DNP-lysine-Sepharose. After washing with PBS the IgG was eluted with 50 mM DNP-glycine pH 7.2, which was subsequently removed on a Dowex 1X8-400 column equilibrated in PBS.

For the protein A (SpA) -Sepharose chromatography the buffer system used was 0.05 M citric acid / 0.1 M Na₂HPO₄ / BSA (1 mg/ml) (CPB). The BSA (which does not interact with SpA) was found to be necessary to prevent non-specific adsorption of the IgG at such low concentrations to the glassware etc. The column of SpA-Sepharose (16 mm x 4.4 mm) was equilibrated with CPB pH 8 and the labelled IgG loaded in the same buffer. After washing with this buffer, the column was eluted with a linear gradient of CPB pH 8 to CPB pH 3 (25 ml + 25 ml) at a flow rate of 30 ml/hour. The fractions (0.5 ml) were counted in a

tolulene-based scintillant.

6.2.3 Removal of bound hapten from IgG

As the Clq binding and Cl activation assays required that the IgG bind hapten, it was necessary to ensure that all traces of bound DNP-glycine were removed. This was achieved by exchange dialysis against dinitrophenol (0.05 M) / PBS pH 7.2 as described in section 4.2.2.

6.2.4 Clq binding

This was performed as described in chapter 4 with the following exceptions: the final concentration of IgG/DNP-Affigel 701 was 0.6% (v/v); the total assay volume was 100 μ l; addition of Clq and removal of supernatant fractions were with Hamilton syringes to improve precision.

6.2.5 Cl activation

This assay used the method of N. R. Richardson & A. Feinstein (personal communication), which is based on the Clq binding assay described in chapter 4 and the Cl activation assay of Folkerd *et al.* (1980). The following protocol was followed. All tubes were pre-soaked overnight in 1% BSA / BBS / NaN_3 . IgG in PBS was incubated overnight with DNP-Affigel 701 (5 μ l total volume of beads). The IgG/DNP-Affigel was then pelleted by centrifugation, washed by resuspension/centrifugation in BBS / NaN_3 , and finally resuspended in 225 μ l of 0.05% BSA / BBS / NaN_3 on ice. To this mixture 25 μ l of an ice-cold [^{125}I]-Cl solution (in 0.3 M NaCl / 10 mM Na acetate / 1% BSA / 1 mM CaCl_2 pH 5.1,

centrifuged for 10 minutes at 12,000 x g prior to use) was added to give a Cl concentration in the final mixture of ~ 1 µg/ml. After 30 minutes incubation on ice to allow equilibrium to occur a 40 µl aliquot was removed and added to 10 µl of 1 mM NPGb solution on ice to stop any subsequent activation of Cl. This '0 minutes' point was then centrifuged to pellet the IgG/DNP-Affigel 701 + bound Cl, and the entire supernatant was removed and transferred to a separate tube. After removal of this '0 minutes' point the reaction tube was transferred to a 37°C water-bath to allow activation to proceed, and at various times aliquots were removed and processed as the '0 minutes' point. After all the samples had been taken, the supernatant and pellet fractions were prepared for PAGE by addition of 2-mercaptoethanol-containing SDS sample buffer.

The extent of Cl activation in the supernatant and pellet tubes was determined as described by Folkard et al. (1980): the supernatant and pellet fractions were analysed by SDS-PAGE (Laemmli, 1970) using 10% acrylamide gels. The gels were fixed in acetic acid / methanol and dried, positions of the labelled protein bands were determined by autoradiography using intensifying screens and pre-flashed X-ray film (Laskey & Mills, 1975). The intensity of each band was estimated by densitometry of the developed film, and the percentage activation expressed as:

$$\frac{(\bar{C}l_r [H] + \bar{C}l_s [H]) + (\bar{C}l_r [L])}{(Cl_r + Cl_r) + (\bar{C}l_r [H] + \bar{C}l_s [H]) + (\bar{C}l_r [L])} \times 100\%$$

where [H] and [L] denote the heavy and light chains of Cl_r or Cl_s produced after activation (proteolytic cleavage). Fig. 6.1 shows the results of a typical experiment using IgG2a(K3)/DNP-Affigel 701. At zero time there is little activation; with increasing time the intensity

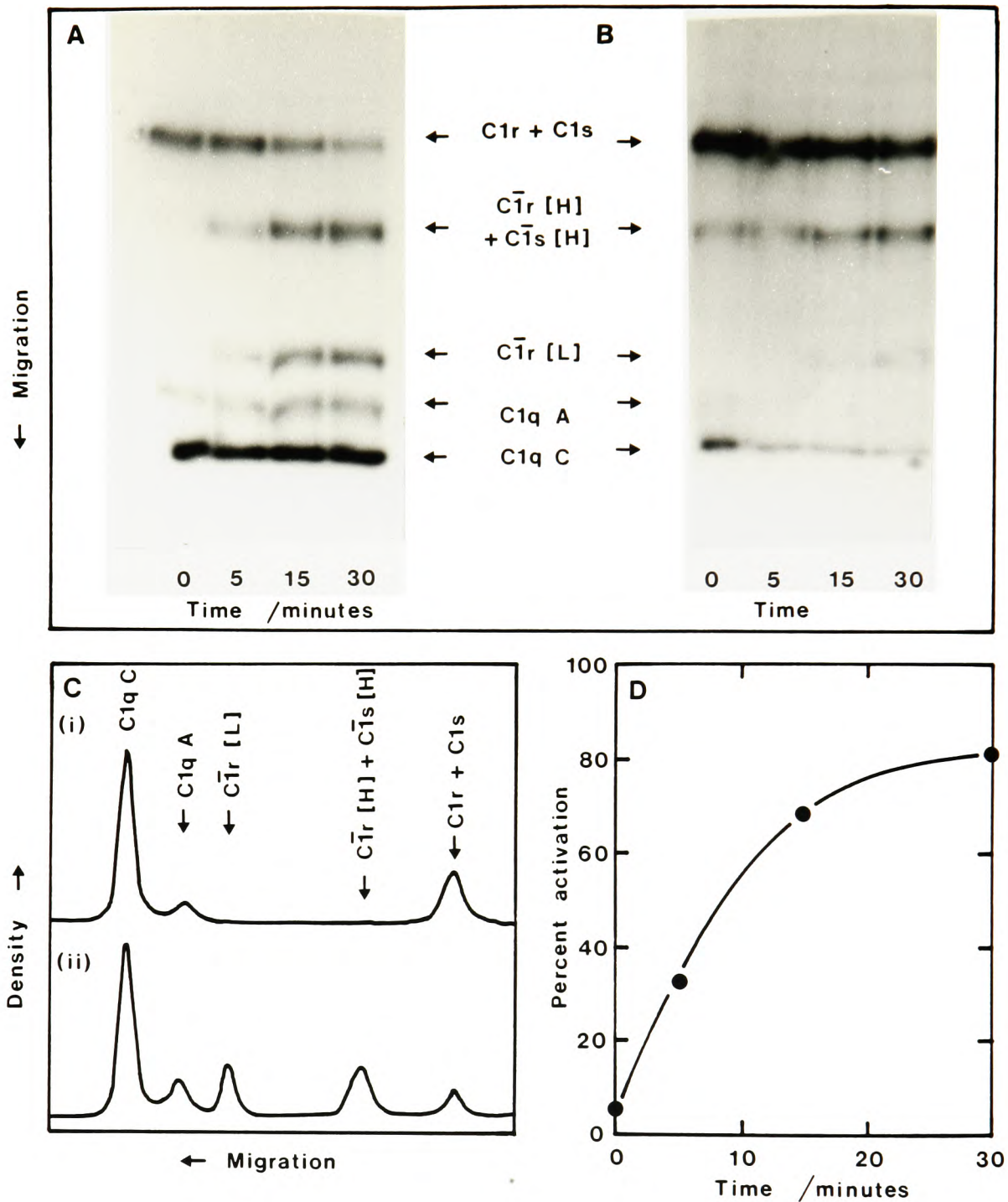


Figure 6.1 Cl activation by IgG2a/DNP-Affigel 701 at 37°C

(a) Autoradiograph of SDS-PAGE of [¹²⁵I]-Cl to show activation of bound Cl (pellet fraction)

(b) As (a) but showing activation of Cl in the supernatant fraction

(c) Densitometric scan of the 0 minutes and 30 minutes time points of (a)

(d) Percentage activation of the bound Cl (a) with time

of the (Clr + Cls) band decreases, with concomitant increase in the (C \bar{l} r [H] + C \bar{l} s [H]) and (C \bar{l} r [L]) bands. It is apparent that activation of the bound Cl (i.e. in the pellet fraction, Fig. 6.1a) is more rapid than that of the unbound Cl (that in the supernatant fraction, Fig. 6.1b). It is also clear that there is selective binding of the Clq to the IgG/DNP-Affigel (Clq iodinated preferentially in the C chain [Heusser et al., 1973; Tenner et al., 1981]). Fig. 6.1c shows densitometric scans of two tracks of Fig. 6.1a which indicate how it is possible to quantitate the activation of the Clr and Cls from the relative areas of the peaks. The percentage activation versus time for the profiles in Fig. 6.1a is shown in Fig. 6.1d. For brevity only this type of plot (d) will be shown in the results section.

6.2.6 Peptic digestion of IgG

IgG (glycosyl or aglycosyl prepared as in chapter 5) at 40 μ g/ml was digested with 1:50 w/w pepsin at 37 $^{\circ}$ C in 0.1 M acetic acid / sodium acetate pH 4.5 after pre-incubation for 30 minutes under the same conditions without pepsin. The digest also contained [3 H-leu]-IgG (glycosyl or aglycosyl as appropriate) prepared as described in section 5.2.8. Aliquots (20 μ l) were taken at various intervals and the digestion stopped by addition of 3 M tris (3 μ l) to raise the pH. Samples were prepared for SDS-PAGE under non-reducing conditions, and electrophoresis followed by fluorography was performed as described in section 5.2.7. The total radioactivity applied per track was: glycosyl IgG, 18,000 dpm; aglycosyl IgG, 14,000 dpm. Quantitation of the amount of intact IgG remaining at each time point was by densitometry using a Vitatron densitometer.

6.2.7 Binding of IgG to monocyte Fc receptors

The assay used was that of J. Woof & D. R. Burton (personal communication), which is based on assays described by Unkeless & Eisen (1975) and Segal & Hurwitz (1977) but uses a much reduced assay volume. This is greatly advantageous if minimal use of protein material is required. In the assay [125 I]-IgG is displaced by varying amounts of unlabelled IgG. Human lymphocytes were isolated from freshly-drawn heparinized blood by use of a Lymphoprep (Pharmacia) discontinuous density gradient essentially as described in section 5.2.4. They were then washed with RPMI 1640 / FCS and incubated in plastic petri dishes at 37°C / 5% CO₂ for 45 minutes. The adherent monocytes were harvested by washing in 1:1 BSS[-Ca,-Mg] / EDTA (10 mM) : RPMI 1640 / FCS, and counted using a haemocytometer. They were then suspended at 10⁸ ml⁻¹ in BSS[-Ca,-Mg] / BSA (0.2%) / NaN₃ (0.1%) (the azide is present to prevent phagocytic processes [Segal & Hurwitz, 1977]). 10 µl of this suspension was added to 5 µl of [125 I]- human IgG (4 nM in BSS[-Ca,-Mg] / BSA / NaN₃) and 5 µl of test IgG solution (various dilutions). Both the iodinated IgG and the test IgG stock solutions had been ultracentrifuged at 100,000 x g for 30 minutes before use to remove any aggregates. The reaction tubes were then incubated for 2 hours at 37°C. After this time the monocytes were resuspended and 2 x 8 µl aliquots were removed from each tube. These aliquots were layered over 10 µl Versilube (Alfa) which had in turn been layered over 10 µl of 1:2 Lymphoprep : BSS in siliconized capillary tubes. The monocytes, together with any bound IgG, were separated from the unbound IgG by centrifuging them through the Versilube / Lymphoprep layers. The tubes were then snapped to separate the pellet and supernatant fractions, and the amount of [125 I]-IgG in each was determined. As well as varying amounts of test

IgG, the following samples were also included: [^{125}I]-IgG alone, to give the initial binding; a large excess of unlabelled IgG, to give the non-specific background binding. In control experiments (not shown) it had been established that mouse IgG2a (K3) inhibited the binding of human [^{125}I]-IgG in exactly the same manner as unlabelled human IgG. The amount of protein in the stock IgG samples after ultracentrifugation was determined by the Bio-Rad dye-binding protein assay (micro version) using dilutions of an IgG sample of known concentration to construct the standard curve.

6.2.8 Molecular graphics

Molecular graphics were generated on an Evans & Sutherland graphic display system run by a Digital PDP 11/70 computer (department of Molecular Biophysics, Oxford), using the programs FRODO (Jones, 1982) and FITZ (Taylor, 1983). Superposition of protein structures was by the program FITZ which utilizes a non-linear least squares routine to minimize the root mean square (RMS) deviation of specified alpha-carbon atom co-ordinate sets. Figures were generated by direct photography of the display screen using coloured filters. The co-ordinate sets used were: Fab', NEW (Saul et al., 1978); Fc, rabbit (B. J. Sutton & D. C. Phillips, unpublished data) and human (Deisenhofer, 1981).

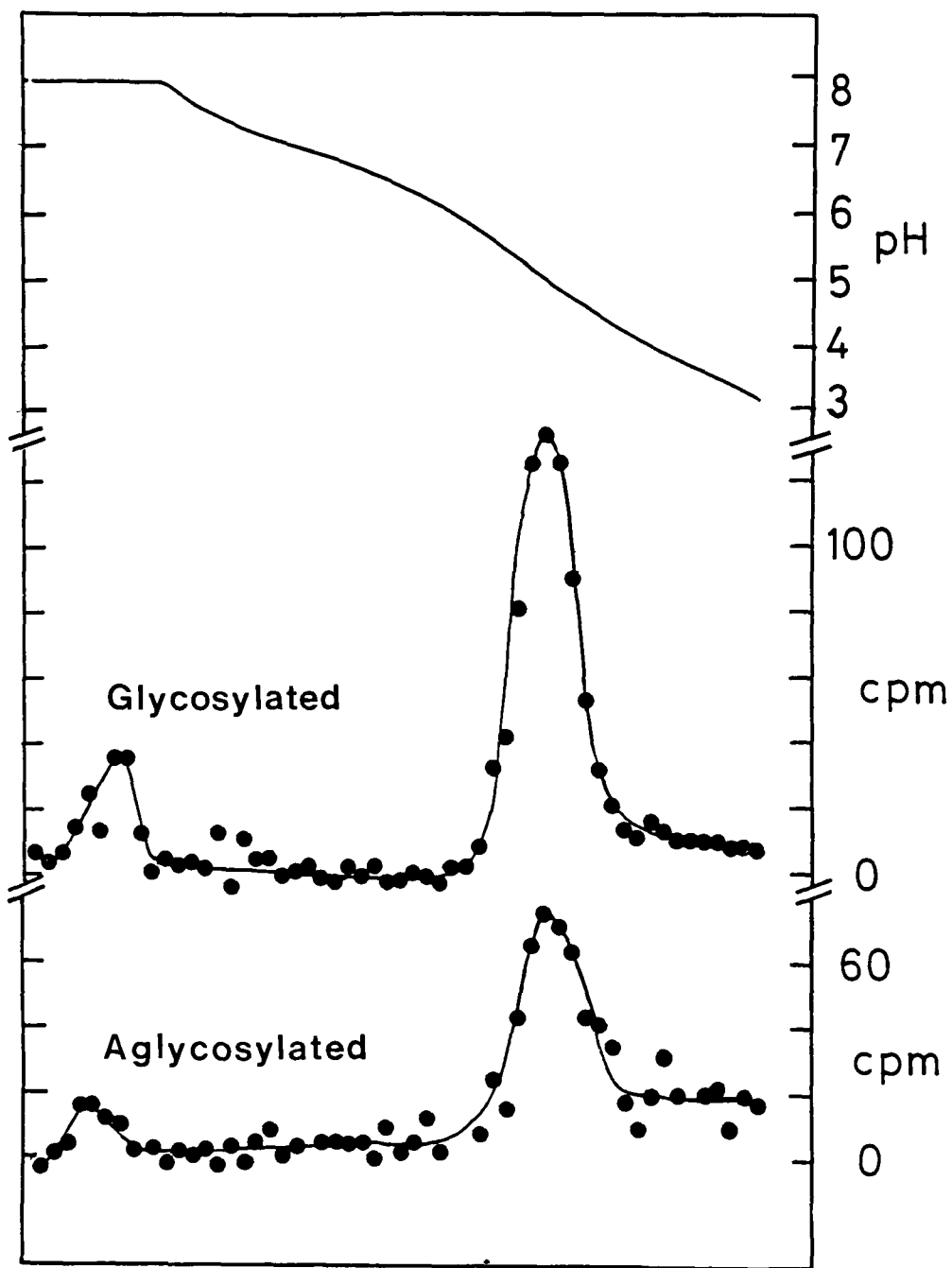


Figure 6.2 The binding and pH elution profiles of radiolabelled glycosyl and aglycosyl IgG to protein A-Sepharose.

6.3 RESULTS AND DISCUSSION

6.3.1 Binding of glycosyl and aglycosyl IgG to protein A

The availability of radio-labelled aglycosyl IgG allows an assessment of its ability to bind to protein A (SpA). The binding / elution profiles of the glycosyl and aglycosyl forms of K3 IgG to SpA-Sepharose are shown in Fig. 6.2. Both IgGs are found to bind equally well to the column. Moreover, both glycosyl and aglycosyl proteins elute with the gradient in an identical manner, at the expected position for a mouse IgG2a (Ey et al., 1978).

These binding studies show that SpA-Sepharose may be used for purification of aglycosylated IgG in the same manner as for the parent IgG, if required. Furthermore, since aglycosylated IgG binds to SpA in a manner which is indistinguishable from normal IgG, this indicates that the conformation of the C_H2-C_H3 domain interface as sensed by protein A remains the same upon aglycosylation. It should be noted however, that structural alterations or destabilization (Huber, 1980; Marquart & Deisenhofer, 1982) localized to the immediate area of the carbohydrate would probably not be detected.

The protein A binding site (see Fig. 1.6) is formed by the unique longitudinal interaction of the C_H2 and C_H3 domains of IgG, where loops b1 and b5 of the C_H2 interact with b6 of C_H3 (for nomenclature see Fig. 1.4b). Any disruption of this quaternary interaction would be expected to result in a loss of binding (individual C_H2 and C_H3 domains do not retain any affinity for protein A [Lancet et al., 1978]). Therefore it is implied that the characteristic orientation of the C_H2 and C_H3

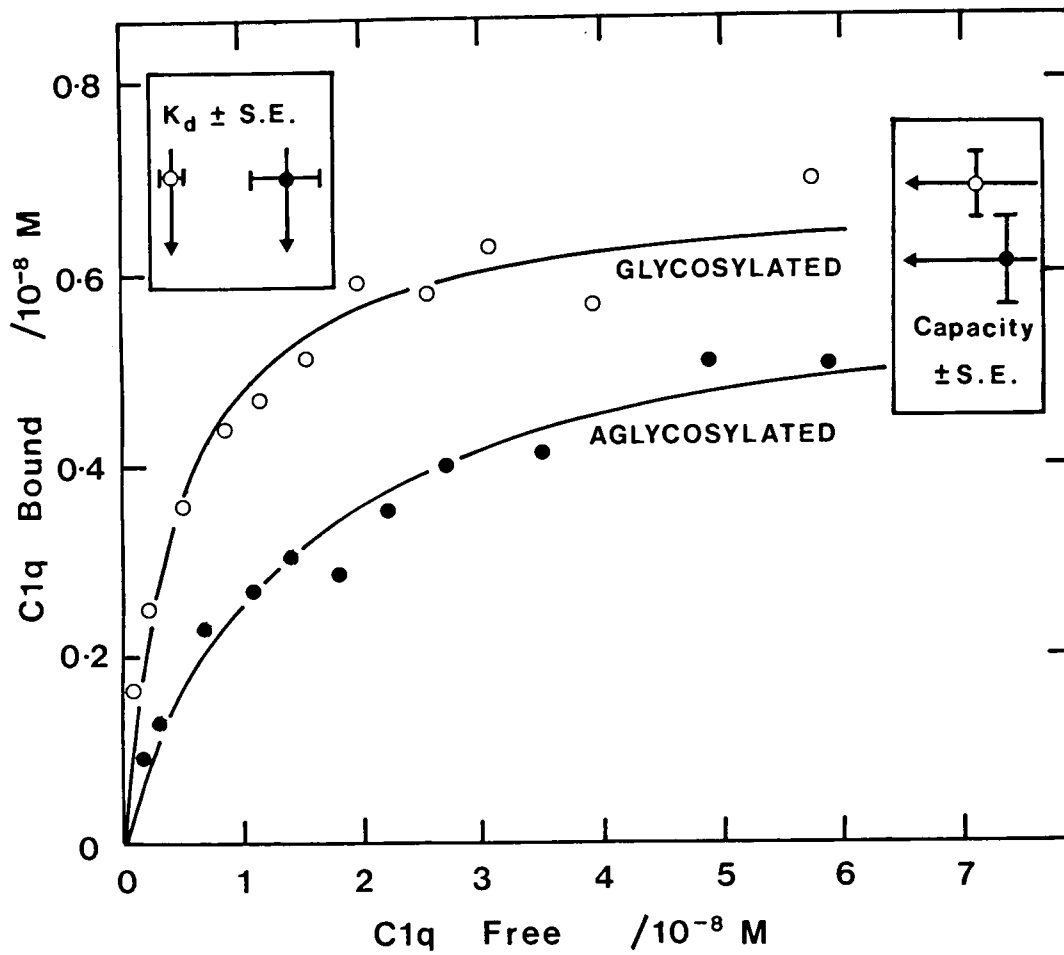


Figure 6.3 Binding of [¹²⁵I]-C1q to glycosyl- (O), and aglycosyl- (●) IgG/DNP-Affigel 701. The curves represent the best fit to the data:

	Cap	± S.E.	Kd	± S.E.
	/nM		/nM	
Glycosylated IgG	6.8	± 0.3	4.3	± 0.9
Aglycosylated IgG	6.0	± 0.5	14.0	± 3.0

domains found in the glycosylated Fc is retained in aglycosyl IgG.

6.3.2 Binding of Clq to glycosyl and aglycosyl IgG

The binding of Clq to glycosyl- and aglycosyl- IgG/DNP-Affigel 701 is shown in Fig. 6.3. It is found that within error both systems have the same capacity to bind Clq. There is a slight difference however in the dissociation constants, the aglycosyl IgG binding Clq three-fold weaker than the glycosyl IgG. Although this difference is outside the error of the assay, it is unlikely that such a small change is of physiological relevance as at serum Clq concentrations ($\sim 15 \times 10^{-8}$ M) no significant difference in binding would be observed. Indeed, similar differences are found between different complement-fixing IgG subclasses (see for example Fig. 4.4). It is therefore concluded that the Clq binding site is still present in aglycosyl IgG and so the carbohydrate cannot be a fundamental part of this site. This result is again indicative that the C_H2 domain structure is not grossly altered in the aglycosyl IgG.

The slight difference in binding constant which occurs when the oligosaccharide is absent leads to differences in binding being most noticeable at low Clq concentrations. Since it is usually at low effective Clq concentrations that complement fixation assays are performed, this may lead to the differences noted by other workers when assaying their carbohydrate-depleted IgG (see section 6.1.3). This indicates the necessity of performing a complete binding curve if a meaningful comparison of binding ability is to be made. It should be noted that the ability to compare these IgGs is also a consequence of the suitability of the assay system used (chapter 4) which is intended

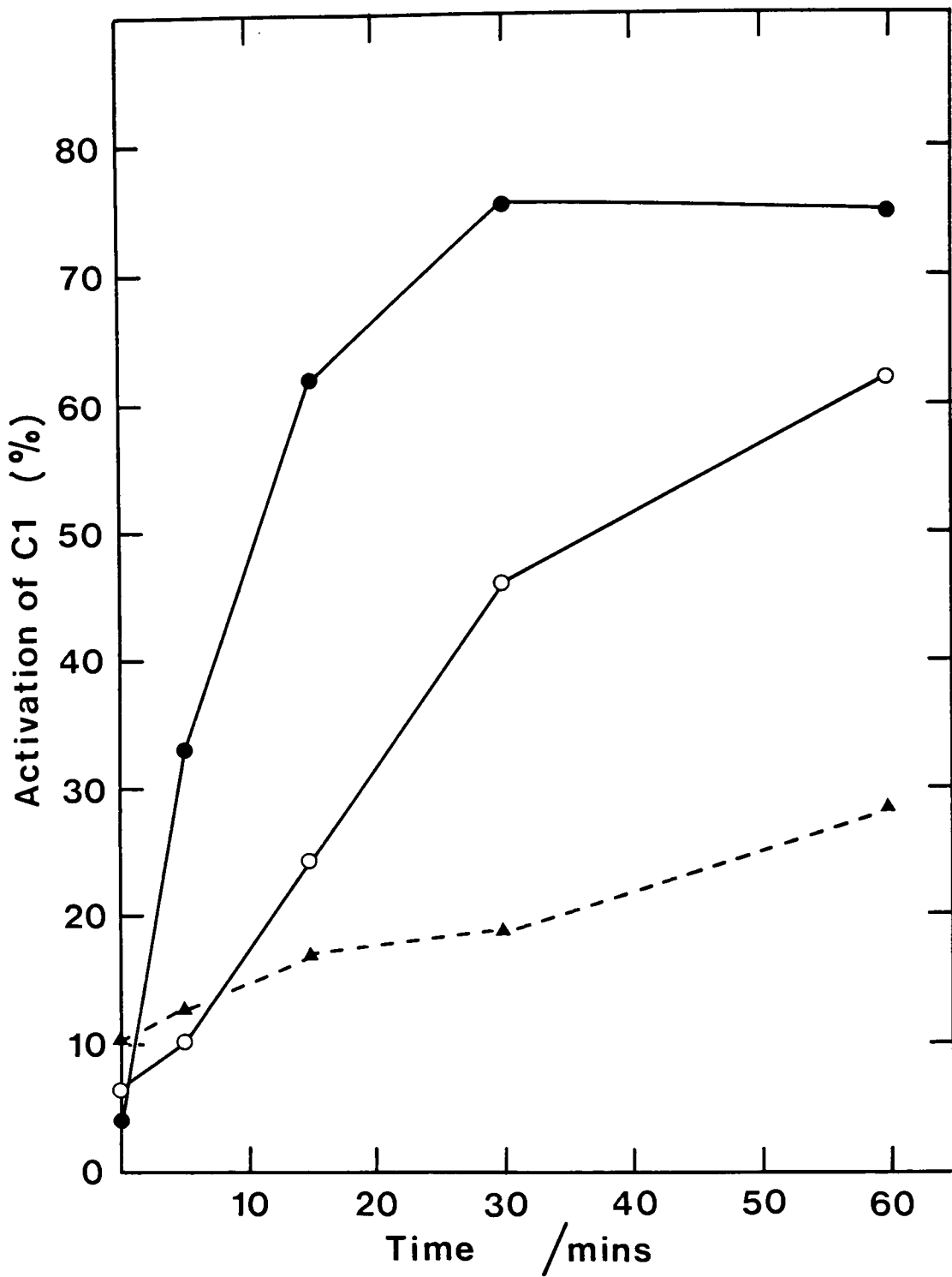


Figure 6.4 Time course of activation of C1 by:

- Glycosyl IgG/DNP-Affigel 701
- Aglycosyl IgG/DNP-Affigel 701
- ▲ DNP-Affigel 701 alone

to eliminate artefacts caused by immune complex lattice formation. The success of this is reflected in the equal capacities for Clq found with both the glycosyl and aglycosyl IgGs. In contrast a comparison of Clq binding to native and glycosidase-treated IgG immune aggregates found significant differences in capacity (A. Dodds, G. Wilson, D. A. Rees & R. R. Porter, personal communication). Such changes probably reflect lattice differences between the different immune complexes, which make the relative Clq binding ability of these preparations difficult to evaluate exactly.

6.3.3 Activation of C1 by glycosyl and aglycosyl IgG

As was described in the previous section, aglycosyl IgG still retains the capability to bind Clq. Binding of Clq alone, however, is not in itself sufficient to activate C1. Both tryptophan-modified (Allan & Isliker, 1974a,b) and glutaraldehyde cross-linked (Folkerd *et al.*, 1980) IgG retain the ability to bind Clq and yet do not activate the bound C1. Therefore it is conceivable that aglycosyl IgG may bind and yet not activate C1, and so its ability to perform this activation was examined.

The time-course of C1 activation (cleavage of C1r and C1s) at 37°C by glycosyl- and aglycosyl- IgG/DNP-Affigel 701, and by a control sample of DNP-Affigel 701 alone is shown in Fig. 6.4. Both IgGs are found to cause significant activation of the bound C1 with respect to the control sample (a further control of C1 alone showed an identical activation profile to C1 + DNP-Affigel 701 [not shown]). Although the rate of activation of C1 by the glycosyl IgG is greater (~ 3 fold) than the aglycosyl IgG, this is to some extent explained by the aglycosyl IgG

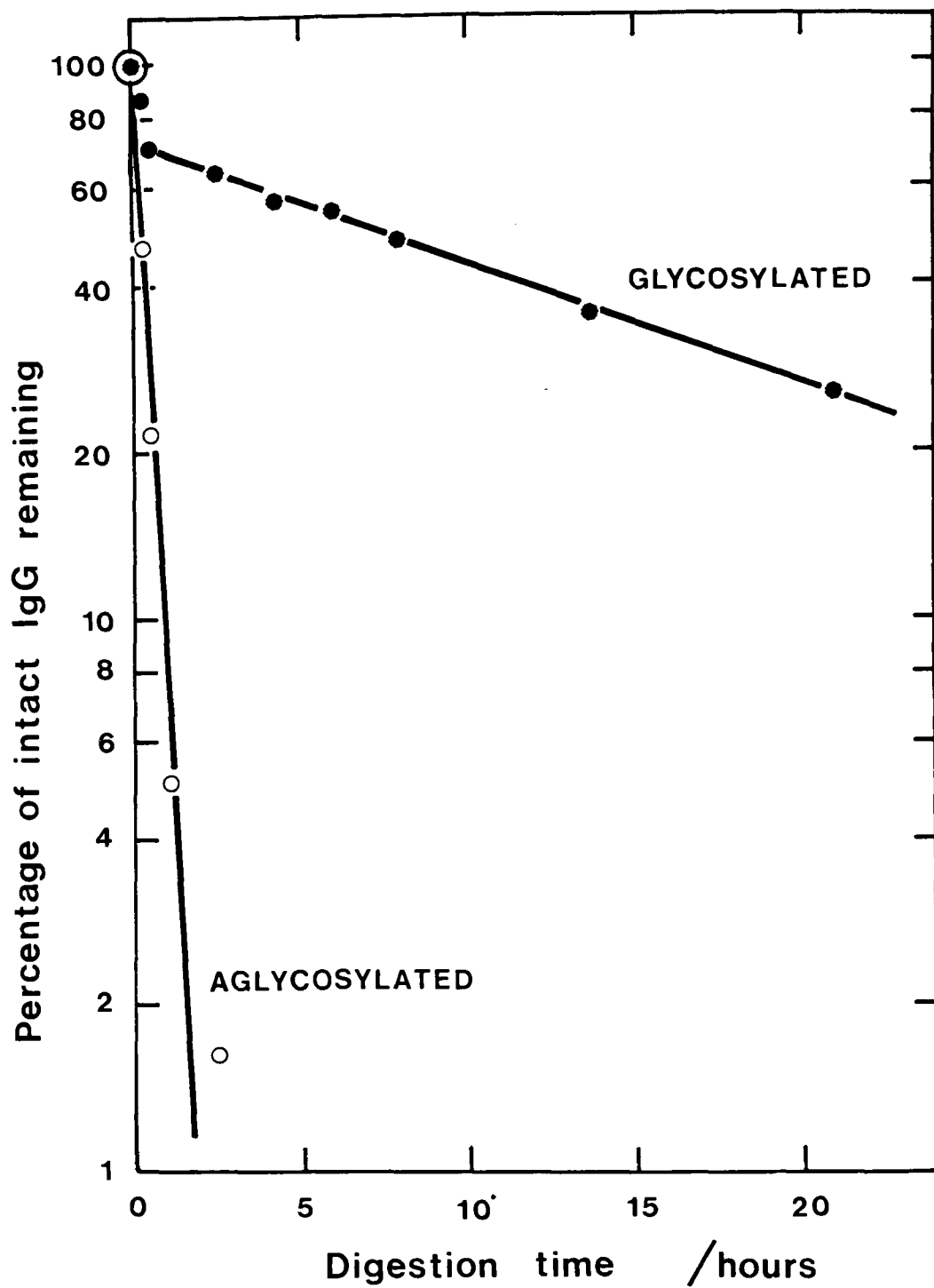


Figure 6.5 Time course of pepsin digestion of glycosyl and aglycosyl IgG.

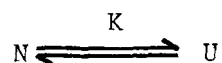
binding less Cl (20% Cl specifically bound compared with 34% to the glycosyl IgG). The difference in binding is expected from the Clq binding results of the previous section and the necessarily low Cl concentration (~ 1 nM) at which the assay is performed. The work of Hughes-Jones & Gorick (1982) has shown that the amount of Cl(q) bound affects the rate of activation of the Cl, presumably due to a proximity effect. Tschopp et al. (1980a) also show that activation rate is dependent on the overall binding constant. In conclusion, the definite activation of Cl by the aglycosyl species rules out the oligosaccharide in playing any major role in either the binding or activation of this component.

6.3.4 Proteolytic susceptibility of glycosyl and aglycosyl IgG

As described in section 6.1.2, it is often found that carbohydrate-depleted proteins exhibit increased proteolytic susceptibility. The time-course of degradation of IgG \pm oligosaccharide by pepsin is shown in Fig. 6.5. Cleavage with pepsin occurs just below the hinge disulphide and at multiple sites within the C_H2 domain, and therefore allows an assessment of the stability of this region. From the figure it is apparent that there is a large increase in the degradation rate when the oligosaccharide is not present. Under the conditions used the half-life of glycosyl and aglycosyl IgG are approximately 14 hours and 14 minutes respectively. This is not due to the presence of additional protease activity in the aglycosyl preparation as during the initial thirty minute pre-digestion period no degradation was observed. With both glycosyl and aglycosyl IgG the fragmentation pattern was identical (formation of a $\sim 100,000$ M_r F(ab')₂ fragment and low molecular weight products which ran with the ion front

in the 9% acrylamide gel used [i.e. <20,000 M_r - presumably pFc' and small peptide fragments])

Since the rate of proteolysis depends on the accessibility of cleavage points the increased proteolysis of aglycosyl proteins has almost invariably been explained by suggesting that the oligosaccharide acts to sterically "shield" these cleavage points (Beeley, 1977; Marshall, 1978; Montreuil, 1980). There is however another mechanism available for altering the proteolytic susceptibility of a protein. It is established that proteolytic enzymes do not generally act on the "native" state (N) of a protein but on an "unfolded" form (U) (Matthyssens et al., 1972; Imoto et al., 1974; Lapanje, 1978; Privalov, 1979). Thus in the simplest case for a single equilibrium:



the rate of proteolysis is proportional to the amount of U, and therefore reflects the equilibrium constant, K, between the two forms. Alterations to K, i.e. to the conformational stability of the molecule, will result in alterations in the rate of proteolysis. This effect accounts for the reduced proteolytic susceptibility of thermostable proteins (Daniel et al., 1982). Using the treatment of Matthyssens et al. (1972) the sixty-fold increase in proteolytic rate between glycosyl and aglycosyl IgG indicates a sixty-fold increase in the N \rightleftharpoons U equilibrium constant, which would be accounted for by a reduction in the stability of the N form by 10 kJ mol⁻¹. Therefore the increased proteolysis of aglycosyl IgG may indicate reduced stability of the C_H2 domain in the absence of carbohydrate. It should be noted that a change in the stability of a protein is not necessarily reflected in the intrinsic protein conformation as determined by X-ray

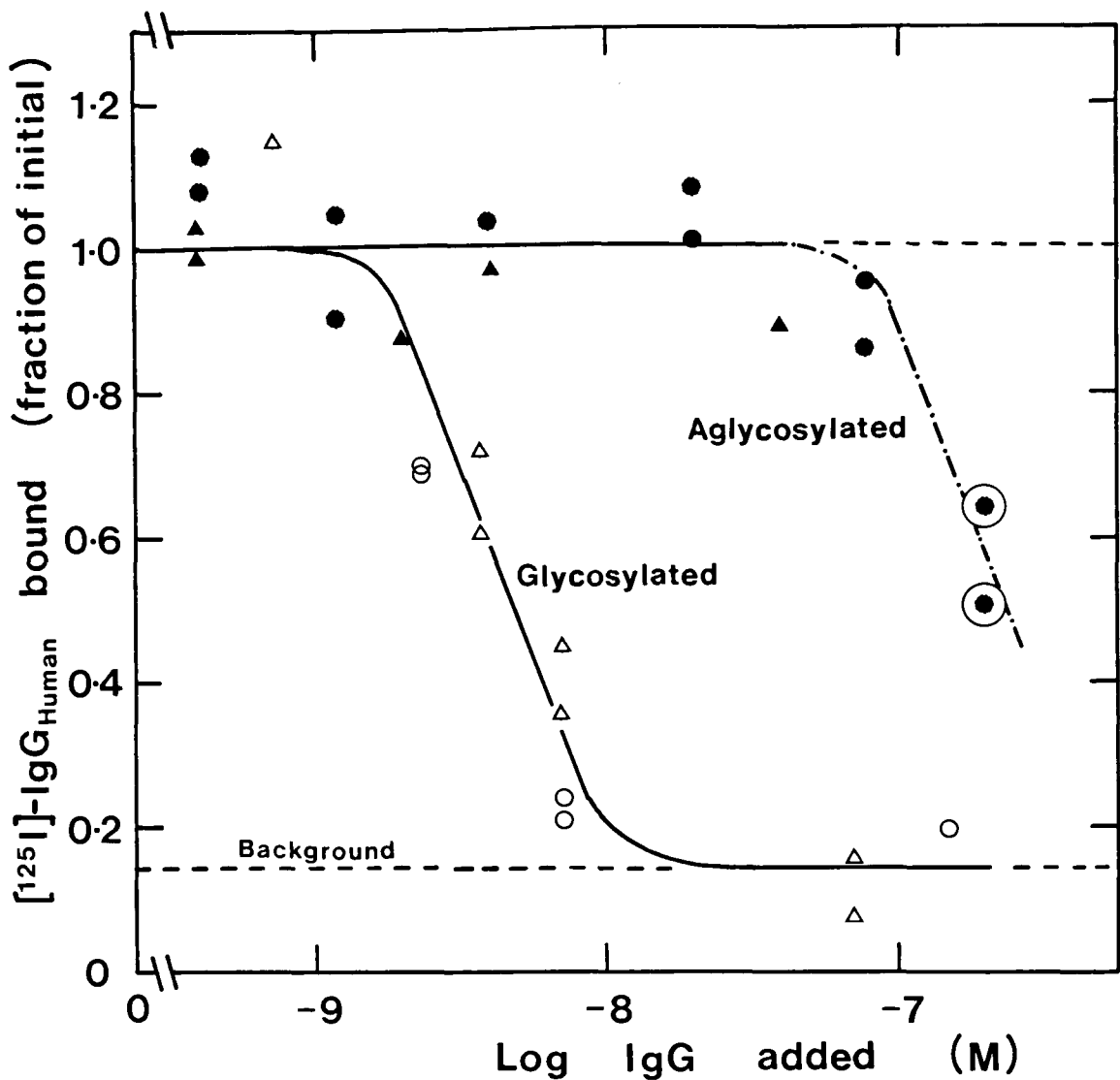


Figure 6.6 Inhibition of binding of human [^{125}I]-IgG by glycosyl and aglycosyl IgG. The figure is a compilation of two separate experiments (denoted by circles and triangles). The initial percentage [^{125}I]-IgG bound were: ▲, 13.8%; ●, 11.1%. The points marked ⊙ were performed using an uncentrifuged preparation and therefore may contain aggregates which would cause greater inhibition than an aggregate-free preparation of the same concentration.

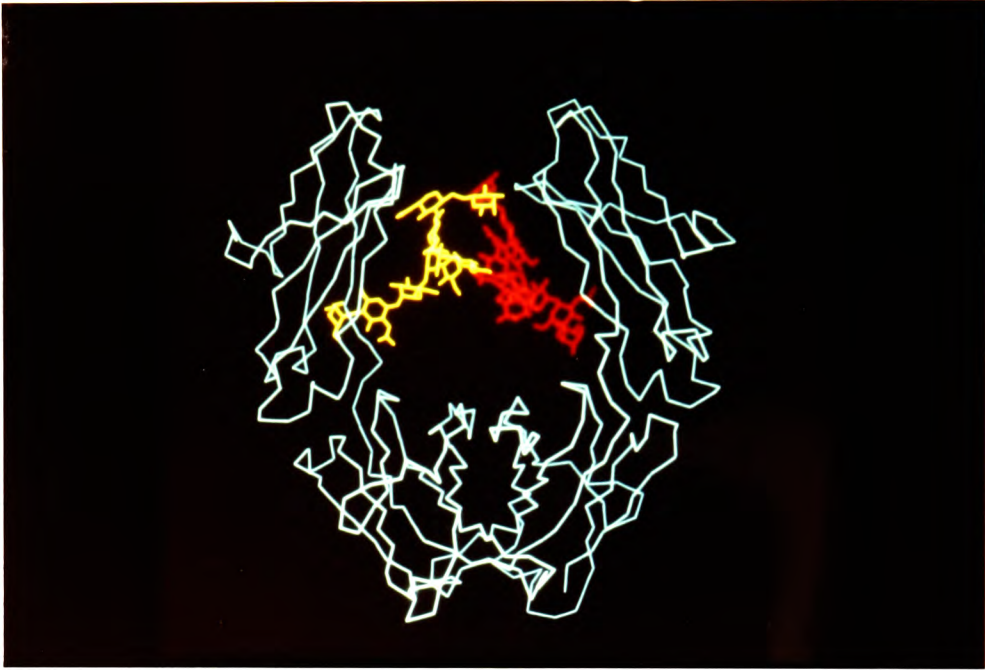
crystallography (Grütter et al., 1979).

6.3.5 Effect of aglycosylation of IgG on binding to monocytes

The ability of glycosyl and aglycosyl IgG to inhibit the binding of labelled human IgG to human monocytes is shown in Fig. 6.6. It is found that there is at least a fifty-fold difference in the inhibitory capacity (K_{50}) of the two proteins. Since the level of glycosylated contaminant in the aglycosyl preparation cannot realistically be expected to be lower than 2%, then within the limits of the experiment the aglycosyl IgG displayed no activity. As discussed in section 6.1.3 a similar conclusion can be reached with the results of Koide et al. (1977) for monocyte binding by carbohydrate-depleted rabbit IgG.

Therefore under the conditions of the assay aglycosyl IgG does not bind to monocyte Fc receptors. There are two possible explanations for this phenomenon. Firstly the aglycosyl IgG may have lost the necessary binding site (i.e. the oligosaccharide itself or a structure maintained by its presence). Secondly it is possible that the aglycosyl IgG is degraded during the incubation period. As was shown in section 6.3.4 the susceptibility of IgG to proteolysis is vastly increased upon removal of the carbohydrate. It is well known that cells of the mononuclear phagocyte series secrete proteases (Schnyder & Baggiolini, 1978; Adams, 1981; Werb, 1981) - indeed proteolysis of IgG by macrophages has been observed, and implicated in B cell activation (see section 1.9.4). In order to test the integrity of the IgG under the conditions used a sample of [³H-leu]-IgG was incubated with monocytes under the same conditions used in the assay. At the end of the incubation period the IgG was examined by SDS-PAGE under non-reducing

(A)



(B)

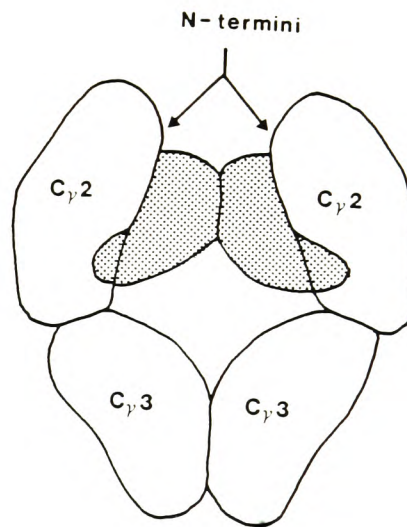


Figure 6.7 The structure of the Fc fragment of rabbit IgG.

(a) The protein alpha-carbon backbone is depicted in blue, the oligosaccharide chains of each chain are shown red and yellow respectively.

(b) Schematic illustration of (a).

conditions and the bands located by fluorography. It was found that no significant proteolysis of aglycosyl IgG had occurred during the assay. It is therefore concluded that the effect observed was due to an inherent lack of interaction with monocyte Fc receptor. This result is extremely interesting, although in light of the limited knowledge of the structural requirements for monocyte binding (see section 1.7.3) is somewhat difficult to interpret. It does however provide further evidence for the involvement of the C_H2 region in binding of IgG to monocyte Fc receptors. While the lack of inhibition by isolated C_H2 domains (Barnett Foster et al., 1980) makes it unlikely that the carbohydrate itself is the sole recognition site, this possibility cannot be ruled out and is presently being investigated. As noted in section 1.7.3 there are several instances where a modification to IgG affects cell binding but not Clq binding, or where a quite subtle alteration to the IgG structure causes significant reduction in Fc receptor binding. In this respect it is tempting to speculate that the structural requirements for Fc receptor binding are quite stringent. This is perhaps reflected in the very tight binding by monomeric IgG (K₅₀ from Fig. 6.6 ≈ 5 nM). The aglycosylated IgG may therefore have lost sufficient structural integrity to affect its interaction with monocyte Fc receptor while its ability to bind protein A and Clq are still retained.

6.3.6 The influence of carbohydrate on the Fc structure

As was discussed in section 1.3.2 the C_H2 domains of IgG are unusual in that they are separated by the Asn-297 oligosaccharide. The resultant Fc structure is illustrated in Fig. 6.7. This arrangement poses the following questions:

(A)



(B)

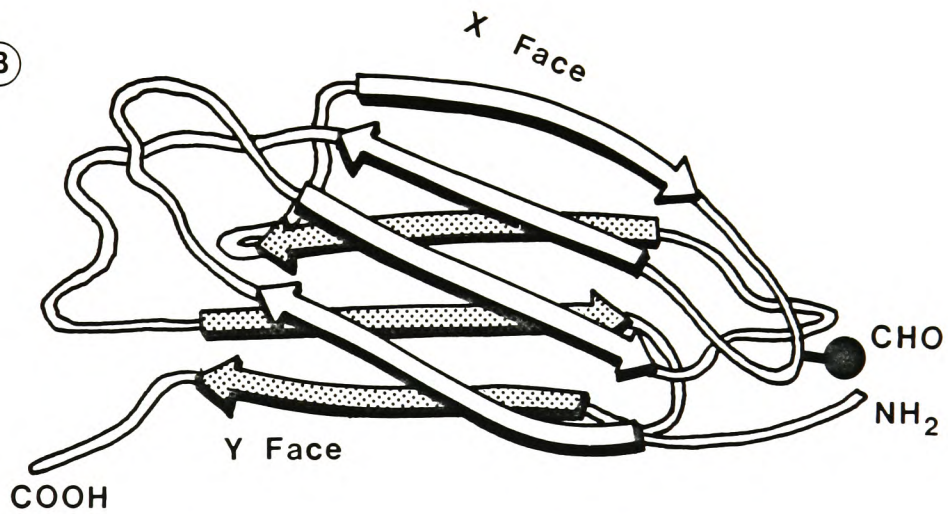


Figure 6.8

- (a) Superimposition of the alpha-carbon atoms of the C_H2 domain (blue) and the C_H3 domain (red) of rabbit Fc.
- (b) Schematic illustration of the domain structure shown in (a) to indicate the positions of the β -sheets and the carbohydrate (CHO) attachment point.

1. Does the carbohydrate influence the structure of the C_H2 domains?
2. Is the unusual C_H2 pairing arrangement a consequence of oligosaccharide attachment i.e. if the carbohydrate were not present would a normal X-X face pairing interaction occur?

It is possible to go some way to answering these questions by an analysis of the Fc structure. This was achieved by computational "model-building". The following discussions relate to the rabbit Fc structure, although since human Fc is essentially identical they are applicable to both structures. In order to determine whether the carbohydrate influences the C_H2 domain conformation, the individual C_H2 and C_H3 domains were compared by superimposition using the program FITZ (see section 6.2.9) (superimposition was using alpha-carbon co-ordinates of homologous β -strands (Beale & Feinstein, 1976), RMS deviation = 2.34 Å on 49 atoms). Although not quite as good a fit is achieved as when comparing C_H1 with C_H3 (RMS difference of homologous β -sheet alpha-carbon atoms = 0.97 Å on 50 atoms) the structural homology is quite good, and far better than a similar comparison of constant and variable domains (see below). The superimposed C_H2 and C_H3 domain structures are shown in Fig. 6.8. No great structural differences are evident between the domains, and therefore addition of carbohydrate does not cause the C_H2 domain to adopt a different tertiary conformation. Any effect of the oligosaccharide seems limited therefore to the quaternary interaction between domains. It is possible however that the presence of an X face interaction with carbohydrate, replacing the normal X face interaction with protein, maintains a correct C-domain conformation which would otherwise be altered.

The effect of the oligosaccharide on quaternary domain

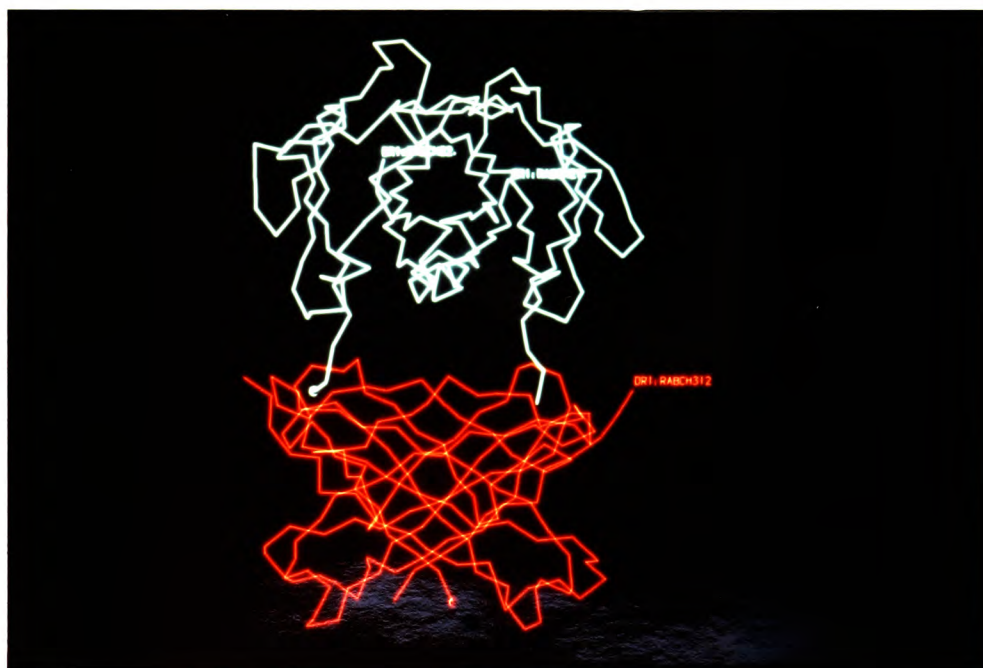


Figure 6.9 Orthogonal views of the two C_H² domains (blue) modelled to interact via their X faces as described in the text. The (C_H³)₂ structure is shown in red. The N-termini of the two C_H² domains are arrowed.

interactions was examined in the following manner. At least two constraints are exerted on the quaternary orientation of the C_H2 domains.

1. The hinge disulphide is formed in both glycosyl and aglycosyl IgG (see chapter 5) which limits the N-terminal freedom of the C_H2 domains.
2. The domain is linked to the $(C_H3)_2$ structure, which similarly limits the C-terminal end of the C_H2 . The $(C_H3)_2$ dimer is known to possess an extremely stable structure (Ellerson et al., 1976; Iseman et al., 1979; Sumi & Hamaguchi, 1982) which is independent of the presence or absence of the C_H2 domains (Phizackerly et al., 1979).

It is assumed that any aglycosyl Fc structure must also conform to both the above constraints. Although in normal IgG the Asn-297 carbohydrate would prevent X-X face interactions, this situation may be modelled (as a postulated aglycosyl C_H2 structure) by superimposing the protein part of the individual C_H2 domain structures on the $(C_H3)_2$ part of Fc. When this is performed it is possible to determine whether the resultant arrangement is feasible i.e. whether it can be formed within the limits of the two constraints described above.

The resultant modelled structure is shown in Fig. 6.9. The N-terminal residue shown in the C_H2 domain is Pro-238. In rabbit IgG the inter-heavy disulphide occurs nine residues before this at Cys-229. In mouse IgG1 the distance between the residue at the position equivalent to Pro-238 and the hinge disulphide is only six residues. The distance around the top of the X-X modelled domain-pair between the two Pro-238 residues was found to be $\geq 75 \text{ \AA}$. Although adjacent alpha-carbon atoms in a polypeptide chain are 3.8 \AA apart, the maximum distance actually found over several residues is an average of 3.3 \AA per

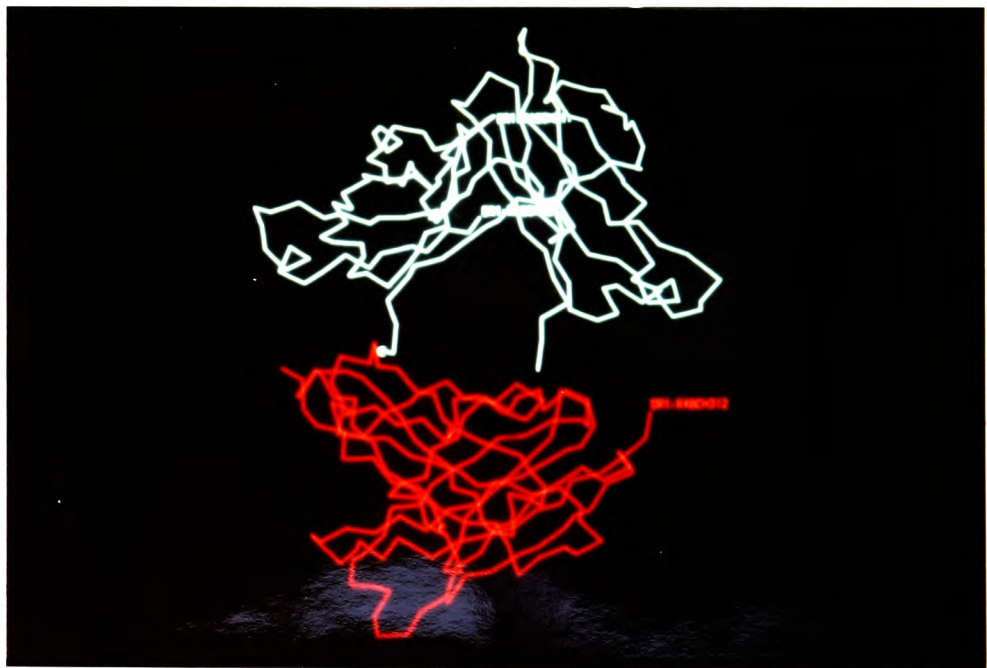


Figure 6.10 Orthogonal views of the two C_H2 domains (blue) modelled to interact via their Y faces as described in the text. The (C_H3)₂ structure is shown in red. The N-termini of the two C_H2 domains are arrowed.

residue in β -sheet structure. Since the sequence 229 to 238 in rabbit IgG contains four proline residues the average distance per residue would be even less than this. It is therefore found that in this modelled X-X C_H^2 arrangement (essentially identical to the $(C_H^3)_2$ region) the N-termini of the two C_H^2 domains are too far apart to allow the formation of the hinge disulphide. Hence such an X-X interaction cannot occur. Model-building to pair the C_H^2 domains via their Y faces was performed similarly by transformation of their co-ordinates onto the V-domains of Fab' NEW (this pairing would not be hindered by the presence of carbohydrate). The superimposition in this case is relatively poor due to the inherent differences between C and V domains (see section 1.3.1) (superimposition of homologous strands: C_H^2 onto V_H , RMS difference = 4.24 Å with 50 atoms; C_H^2 onto V_L , RMS difference = 3.09 Å with 45 atoms). However, the resultant structure (Fig. 6.10) again shows that while joining the C-termini of the postulated $(C_H^2)_2$ structure to the $(C_H^3)_2$ is feasible (as in Fab), the N-termini of the C_H^2 domains are too widely separated to allow formation of the hinge disulphide (the distance from Pro-238₁ to Pro-238₂ across the top of the Y-Y modelled domain-pair is ≥ 65 Å).

This allows the following conclusions. Irrespective of carbohydrate attachment, the two C_H^2 domains are unable to interact to form either the usual C-domain X-X face pairing or the V-domain Y-Y pairing due to the constraint of forming the hinge disulphide. In order for this to form, the C_H^2 domains must adopt an arrangement whereby their N-termini come into quite close proximity (see Fig. 6.7, the distance between the two Pro-238 residues = 19 Å). It is this factor and not the presence of oligosaccharide which necessitates the separation of these domains. A similar argument also applies to the C_H^3

domains of IgM and IgE which must join the X-X paired C_H4 domain to the X-X paired C_H2 domain (Beale & Feinstein, 1976). The consequences of the above arguments from model-building are that aglycosyl IgG is likely to have the same quaternary arrangement of domains as glycosylated IgG. This is substantiated by the functional studies in this chapter which show that Clq binding, C1 activation and protein A binding are not significantly affected by loss of carbohydrate. In particular the continued binding of protein A is strong evidence that the relative orientations of the C_H2 and C_H3 domains are unaltered in aglycosyl IgG. The lack of interaction with monocyte Fc receptor shows however that there are some changes in the structure, though these are not large enough to be sensed by Clq or protein A.

6.3.7 Maintenance of stability of IgG by oligosaccharide

It has been demonstrated that the hydrophobic contribution to the free energy of protein folding can be related to the reduction of surface area accessible to water (Chothia, 1974) so that:

$$\Delta G_h = \gamma \Delta A$$

where ΔG_h is the change in hydrophobic free energy, ΔA the change in accessible surface area, and $\gamma \approx 100 \text{ J/mol} \cdot \text{\AA}^2$. Thus as a globular protein folds it reduces the proportion of surface accessible to aqueous solvent and therefore increases its stability (Chothia, 1975). It has been estimated that it is the hydrophobic energy arising from such effects which provides the major factor in the stabilization of protein-protein and protein-ligand interactions (Chothia & Janin, 1975; Janin & Chothia, 1978a,b).

The considerations of section 6.3.6 have shown that for the C_H2

domain the lateral domain-domain interactions found in all other IgG domains are not available due to inherent constraints of the protein domain structure. The accessible surface area lost upon interaction of two C_H^3 domains is found from the crystal structure (Deisenhofer, 1981) to be 1090 \AA^2 per domain. A similar reduction is expected for the $C_H^1-C_L$ structure. The free energy available from this interaction is unavailable to the C_H^2 domains. Although no longitudinal interactions are found in Fab, they do occur in Fc providing a loss of accessible surface of 400 \AA^2 on each C_H^2 domain and 380 \AA^2 on each C_H^3 domain (Deisenhofer, 1981). Some additional burying of surface residues probably occurs at the N-terminal end of the C_H^2 domain (the crystal structure co-ordinates do not include the extreme N-terminal hinge region) and possibly between C_H^2 and Fab (although this cannot be great because the Fab and Fc regions are essentially thermodynamically independent [Tischenko et al., 1982]). However, these interactions do not fully compensate for lack of the lateral domain interactions, and in the absence of carbohydrate the accessible surface area of a C_H^2 domain will be $500-690 \text{ \AA}^2$ greater than that calculated for a C_H^1 domain. Consequently the C_H^2 domain is expected to be correspondingly less stable. Addition of oligosaccharide covers up the exposed X face surfaces of the two C_H^2 domains "filling up" the area between them. This surface comprises mainly apolar residues, exposure of which would be expected to be highly unfavourable. It should be noted that interactions between carbohydrate and apolar residues seem to be favourable, as judged by such interactions predominating in the combining sites of anti-saccharide antibodies (Gettins et al., 1981). The area covered by carbohydrate is 520 \AA^2 per domain (Deisenhofer, 1981). This brings the water-accessible surface area, and hence the intrinsic stability of the C_H^2 domain to approximately that of the C_H^1

domain. In conclusion, in the absence of carbohydrate the C_H2 domain is expected to display reduced stability. This factor is sufficient to account for the much increased proteolytic susceptibility of aglycosyl IgG.

As was discussed in section 6.1.2 two features of aglycosyl proteins which are commonly observed are increased susceptibility to proteolysis and reduced solubility. In IgG the former is encountered and may be rationalized using thermodynamic arguments - addition of carbohydrate alters the free energy of the folded state without necessarily changing its structure. Reduced solubility or aggregation can also be explained by a similar argument. In this case it may be speculated that interactions between protein molecules could be compensating for the loss of the stabilizing effect of carbohydrate addition.

The proposed stabilizing effect of oligosaccharide addition accounts for many of the general features of aglycosyl proteins (see section 6.1.2) and may be used to explain the results on IgG obtained in this chapter. It may be suggested therefore that stabilization of protein structure is a major reason for protein glycosylation. Protein oligosaccharides do however fulfil other functions - detailed differences in carbohydrate sequence provide for recognition of groups of proteins which are otherwise unrelated in structure, a good example being the lysosomal enzymes. Loss of such a specific recognition marker is likely to be the reason for some of the more specific effects of aglycosylation on certain proteins.

6.3.8 Possible evolutionary significance of IgG glycosylation

It is well-established that immunoglobulins arose by gene duplication. This provides for a linear series of domain structures. In IgM, an earlier evolutionary form than IgG, the Fc comprises three pairs of domains. If as described above the inherent domain structure arising from gene duplication does not allow the formation of similar lateral interactions by adjacent constant region domains, then maximum pairing would leave the middle domain of this Fc region unpaired. Glycosylation of the X face of this domain resulted in its stabilization. Conserving this arrangement after truncation of the $C_{\mu}2$ domain to form the hinge of IgG would leave the $C_{\gamma}2$ domain unpaired, the $C_{\gamma}3$ domain paired. It is known that the integrity of the inter-heavy chain disulphide in IgG is essential for stability (Wright et al., 1978) and maintenance of certain effector functions (Isenman et al., 1975; Haeffner-Cavaillon et al., 1979). This indicates the importance of its formation which possibly substitutes for the missing domain pair. The reason why a new protein structure has not replaced the penultimate immunoglobulin domain is probably explained in terms of the number of mutations required. Production of an Asn-Xaa-Ser/Thr sequence does not require many base changes, does not alter the intrinsic domain structure, and yet by allowing N-glycosylation allows the Fc structure to be stabilized. Changing the protein structure sufficiently to adopt a new stable arrangement would require more base changes and could significantly alter the conformation in areas of the domain required for interaction with secondary effector systems.

6.4 SUMMARY

Aglycosyl IgG is found to retain the antigen-binding, protein A binding, Clq binding and C1 activation abilities of the normal glycosylated molecule. Therefore the carbohydrate cannot be directly involved in any of these binding sites, nor can it be responsible for causing a protein conformation which is necessary for formation of any of these sites. In contrast the aglycosylated IgG is found not to bind to monocyte Fc receptors. This could be indicative of a more subtle structural change on aglycosylation, but in the absence of much evidence on the structural requirements for Fc receptor binding is difficult to interpret unambiguously.

An examination of the Fc quaternary structure shows that the unique "split" C_H2 domain orientation of IgG is not a direct consequence of carbohydrate attachment, but is understood in terms of limitations in the orientations of the domain structures themselves. This leaves the two C_H2 domains unable to form the extensive lateral interactions of the other IgG domains, and results in the aglycosyl structure being significantly less stable in this region, as judged by its greatly increased sensitivity to proteolysis. The carbohydrate acts to cover the otherwise exposed X faces of the C_H2 domains and thereby provides a stabilization of the structure.

It may be suggested that the ability of carbohydrate to reduce the exposed surface area of a protein and thus stabilize the resultant protein structure is an important aspect of protein glycosylation. This effect, together with the possibility of direct "shielding" by the carbohydrate, provides an explanation for the general characteristics of

aglycosyl proteins, namely their enhanced protease sensitivity (reduced intrinsic stability), and their increased tendency to aggregate and become insoluble (i.e. increase their stability by making protein-protein interactions to reduce their water accessible surface). More specific effects of glycosylation can often be understood in terms of direct interactions with the carbohydrate by some receptor system. The information for this seems to be inherent in the primary sequence of the oligosaccharide (e.g. presence of phosphorylated mannose or terminal galactose). The sequence of the IgG carbohydrate from all species so far examined reveals even greater micro-heterogeneity than is usually found for glycoprotein oligosaccharides, perhaps reflecting the constrained environment of the inter-C_H² domain region available to the glycosyl transferases. This may indicate that in this system the oligosaccharide is not in general required for recognition by receptors, or that any receptor which would be involved must recognize a feature of the oligosaccharide which is common to all structures.

APPENDIX 1 - Computer programs

The following computer programs were utilized in this thesis. All programs are written in FORTRAN 77 as implemented on the Digital VAX 11/780 computer.

1. PKA Program to evaluate the best-fit to a single proton titration curve (Henderson-Haselbalch equation) by non-linear regression. The following parameters are obtained: pKa; maximum y value; minimum y value.

2. RINGCALC Program to calculate ring-current shifts using the Johnson-Bovey equation (appendix 2) given x,y,z co-ordinates of the aromatic rings and protons concerned.

3. BIND Program to evaluate the best-fit to a binding curve involving a single dissociation constant (equation 4.1) by non-linear regression. The mathematical basis of the non-linear least-squares technique used in this program and also in program PKA is described by Cleland (1967).

The programs (which contain full documentation in the form of comments within the body of the program) may be found in microfiche form attached to the inside back cover of this thesis.

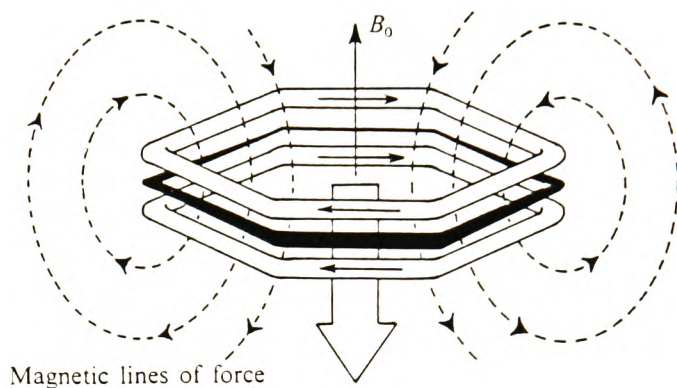


Figure A.1 The ring current and the magnetic lines of force induced in a benzene ring by an external magnetic field B_0 . From Dwek (1973).

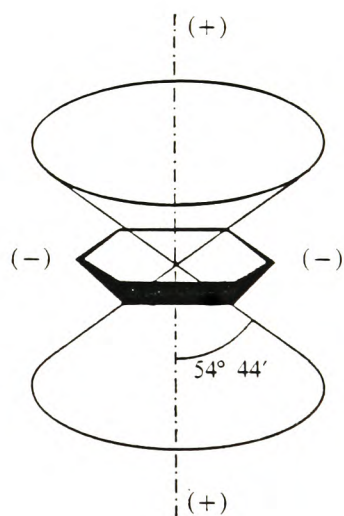


Figure A.2 The shielding and deshielding zones due to the ring current of a benzene ring. The cone separates the two regions. From Dwek (1973).

APPENDIX 2 - Ring-current shifts

The following section provides a brief description of ring-current shifts. For a more comprehensive review see Perkins (1982). In proteins the aromatic rings of His, Phe, Tyr, and Trp residues (and also porphyrin rings in certain proteins) give rise to ring-current shifts. Such effects arise from the circulation of the delocalized π electrons of the ring, giving rise to a local magnetic field which opposes the external applied magnetic field as illustrated in Fig. A.1. Above the plane of the ring a proton will experience an upfield shift as the local field reinforces the external field, in the plane of the ring the shift is downfield (Fig. A.2).

The magnitude of the shift is dependent upon the distance from the centre of the ring, and on geometric considerations. In general for a single aromatic ring the shift experienced:

$$\delta_R \times 10^{-6} = iBG(\rho, z, \varphi)$$

$G(\rho, z, \varphi)$ is a spatial term relating the co-ordinates of the proton to the ring centre. B is a general constant of proportionality and is related to the molecular susceptibility of the aromatic ring. i is an intensity factor which takes into account the specific aromatic ring in question, and will thus differ for each of the aromatic amino acids. For benzene $i = 1$.

Three main approaches have been used to calculate ring-current shifts. These are the classical dipolar model of Pople (1956), the semi-classical current-loop model of Johnson & Bovey (1958) and the quantum mechanical model of Haigh & Mallion (1971). The magnitude of the chemical shifts predicted by these treatments are shown in Fig.

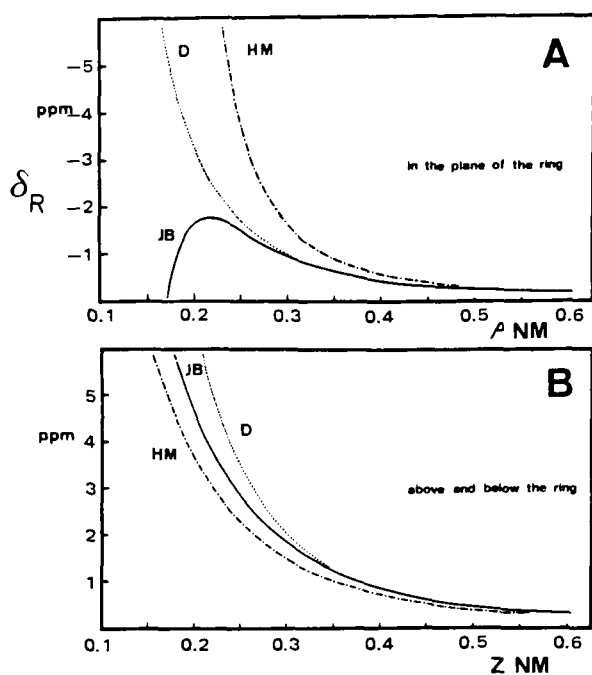


Figure A.3 Plots of calculated ring current shift, δ_R for a phenylalanine ring

(A) in the plane of the ring; (B) above the ring center

From Perkins (1982). D = dipolar equation, JB = Johnson-Bovey equation, HM = Haigh-Mallion equation.

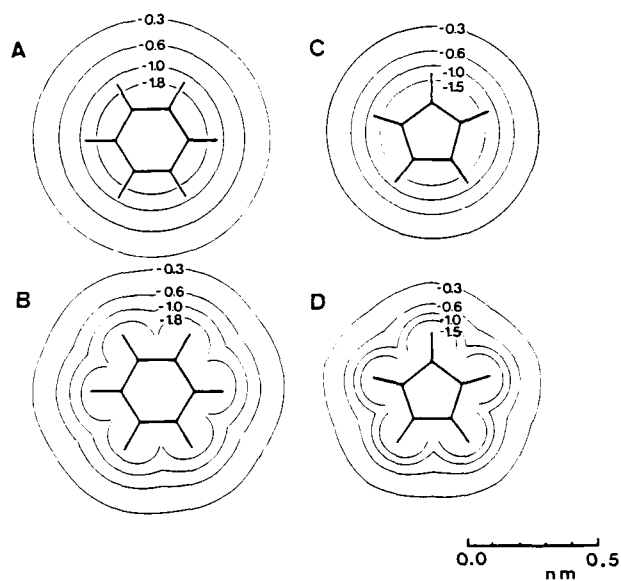


Figure A.4 Contour diagrams illustrating the magnitude of the downfield shift in ppm in the plane of an aromatic ring. From Perkins & Dwek (1980). A, C - Johnson-Bovey; B, D - Haigh-Mallion.

A.3. For applications where it is necessary to calculate the chemical shift change as a proton approaches an aromatic ring (i.e. at distances of > 0.3 nm from the ring centre) the three theories predict very similar shifts. The symmetry of the predicted shifts are shown in Fig.

A.4. The dipolar and Johnson-Bovey models predict cylindrically symmetrical shifts, that of Haigh-Malton does not. The small differences are, however, unlikely to be of any major consequence for biological applications.

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