### Kenneth A. Kelly

Kinetics of Reaction of Antibody and its Subunits with Hapten

Chemistry Ph.D.

A study was made of the equilibrium and kinetic behaviour of the •reactions of antibodies, specific to the 2,4-dinitrophenyl determinant, and their Fab' fragments with hapten. In all cases a slightly higher affinity for hapten of Fab' over its parent antibody was found to be reflected mainly in a two-fold increase in the association rate constant, implying a greater accessibility to hapten of the binding site in Fab' compared to intact antibody.

The affinity of polyalanylated heavy chains of antidinitrophenyl antibodies was 60 times lower than the affinity of intact antibodies for hapten. No antibody activity was found in specific light chains. The difference in affinity constants between heavy chain - and antibody - hapten reactions was found, from kinetic measurements, to be due mainly to a 1,000-fold decrease in the association rate constant. These results indicate that the light chains do not participate directly in the antibody-combining site. KINETICS OF REACTION OF ANTIBODY AND ITS SUBUNITS WITH HAPTEN

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### KINETICS OF REACTION OF ANTIBODY AND ITS SUBUNITS WITH HAPTEN

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Kenneth A. Kelly, B.Sc.

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Department of Chemistry, McGill University, Montreal

July, 1970

#### ACKNOWLEDGEMENTS

I wish to thank Dr. Arnold Froese for his guidance and encouragement during the course of this study and especially for his help and criticism in the preparation of this thesis.

I wish to thank also Professor Alec Sehon for his guidance during this study and for his aid in editing this thesis.

I am grateful to Mrs. Teresa Kennedy for her contributions of rabbit antiserum, to Dr. N.A. Attallah for his help in amino acid analyses and to Dr. T.K.S. Mukkur for his assistance in molecular weight determinations by the Andrews technique.

Last but not least, I wish to thank my wife, Hissin, for her perseverance and encouragement during the course of this study.

I am indebted to the Department of Chemistry, McGill U., where this work was initiated and the Department of Immunology, U. of Manitoba, where it was terminated for providing the laboratory facilities necessary for the performance of this work. This investigation was conducted during the tenure of a studenship from the Government of Trinidad and Tobago and was supported by grants from the Medical Research Council of Canada to Professor A. Sehon and Dr. A. Froese. TABLE OF CONTENTS

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### CHAPTER I

#### THE PROPERTIES OF ANTIGENS AND ANTIBODIES

### The Antigen-Antibody Reaction

Recovery from a disease caused by an infectious organism usually leaves the host in a higher state of resistance or immunity to the disease. This acquired immunity is associated with the appearance in the blood serum of antibodies which, when challenged by the infectious organism, react with it and assist in its removal from the circulation of the host. An antigen is any substance which is capable, under appropriate conditions, of inducing the formation of antibodies and of reacting specifically in some detectable manner with the antibodies so induced. The two basic criteria for immunogenicity of antigens are (a) that they are recognized as alien (i.e. not self) by the responding organism, and (b) that they possess a certain minimal molecular size.

In an attempt to elucidate the characteristic features of antigen-antibody reactions, Landsteiner came upon the discovery that the immune response could be directed to chemically welldefined molecules e.g. nitroaniline and arsanilic acid, by covalently coupling them to proteins (1). Such small molecules which do not evoke the formation of antibodies i.e. are not immunogenic, but which react selectively with antibodies of the appropriate specificity, are called haptens. The study of the interrelationships of haptens and their homologous antibodies has been the most important source of

quantitative information about the nature of antibodies and of the antigen-antibody reaction.

### IN VITRO MANIFESTATIONS OF ANTIGEN-ANTIBODY REACTIONS

#### The Precipitin Reaction

The most common detectable in vitro manifestation of the antigen-antibody reaction is the formation of a flocculent precipitate. The amount of precipitate formed on addition of increasing amounts of antigen to a fixed aliquot of antiserum is represented quantitatively by a precipitin curve (Fig. 1) (2). The precipitin curve can be explained qualitatively by the framework theory, assuming that both the antigen and antibody have a valence greater than one per molecule (3, 4). In the equivalence zone, where the amount of precipitate reaches a maximum, there exist cross-linked complexes of antigen and antibody consisting of an alternating and recurring antigen-antibody pattern, which are sufficiently large to be insoluble. On either side of the maximum, in the zones of antigen and antibody excess, the size of these antigen-antibody aggregates decreases with increasing This framework theory has been confirmed by the ultrasolubility. centrifugal studies of Singer (5) and Bradish and Crawford (6).

### Gel Diffusion

This technique was developed in 1905 by Bechold (7) and improved by Oudin (8) and Ouchterlony (9). The technique of double diffusion (9) in two dimensions of antigen and antibody in agar is particularly useful for the study of complicated antigen-antibody



Fig. 1. Precipitin curve.

systems because of its high resolving power and the ease with which one system can be compared with another. The number of lines of precipitate indicates the minimum number of distinct antigenic substances present in the antigen solution. Double diffusion of antigens towards antibodies from opposite ends of a column of gel is another modification which permits the detection of very small amounts of antibody, as little as 3 micrograms of antibody nitrogen per ml of serum (10).

#### Immunoelectrophoresis

A refinement of double diffusion, this technique was introduced by Williams and Grabar in 1955 (11). This method permits the electrophoretic separation of multiple antigenic mixtures along a horizontal gel track. Antibodies are then added to a parallel trough and are allowed to diffuse out towards the antigen, resulting in arcs of precipitate where optimal ratios of antigen and antibody concentrations exist. Each arc corresponds to a single molecule of antigen.

#### Hapten Inhibition

In the presence of a known precipitating system of antigen and antibody, the addition of haptens or univalent antigens may specifically inhibit the formation of an anticipated amount of immune precipitate by combining with the antibody sites in a manner that precludes aggregation of other antibody molecules. This method of specific inhibition has contributed substantially to information about the size and nature of combining sites of antibodies (10).

#### Agglutination

Antigen is adsorbed onto red cells pretreated with tannic acid (12) or covalently coupled to the cell surface through the intermediary of a cross-linking agent such as bisdiazotized benzidine (13). Addition of antibody to these "sensitized" cells causes clumping into aggregates. Quantitative assay of antibody in sera is performed experimentally by the "extinction dilution" method, the titer of an antiserum being the reciprocal of the last dilution of serum that shows positive agglutination.

### Phage Neutralization

This technique is more sensitive, by a factor of  $10^2$  to  $10^3$ , than haemagglutination techniques described above and is applicable to the detection of anti-hapten antibody of concentrations as little as  $2 \times 10^{-2}$  µg/ml. Hapten molecules are covalently coupled to bacteriophage. When antibody specific to the hapten is added to the hapten-bacteriophage conjugates, it binds to the hapten molecules thus covering the phage surface and neutralizing its activity to lyse bacteria (14). The specificity of the hapten-antibody reaction is demonstrable by the inhibition of neutralization with structurally related haptens.

### THE PURIFICATION OF ANTIBODIES

The procedures used for the isolation of antibodies may be divided into two groups: (a) non-specific methods of fractionation of antisera on the basis of the physico-chemical properties common to immunoglobulins, and (b) specific methods taking advantage of the

specificity of the antigen-antibody reaction.

The most commonly used non-specific methods are: (a) salt precipitation with sodium sulfate (15) or ammonium sulfate (16) which fractionates the serum proteins according to their solubilities, and (b) chromatography on ion-exchange resins (17) or zone electrophoresis (18) which separates the serum proteins according to their charge. These methods give an enriched antibody fraction of good yield but low purity in terms of per cent antibody.

In specific purification methods, insoluble antigenantibody complexes are formed and washed free of contaminating proteins; the complex is then dissociated and the antibody separated from the antigen. Dissociation of the complex can be accomplished using non-specific techniques based on the electrostatic nature of the antigen-antibody reaction such as lowering of pH or salting-out procedures. Separation of the antibody from the antigen can be brought about by insolubilization of the antigen with streptomycin sulfate in the presence of excess hapten (19) or by chromatography on diethylaminoethylcellulose (20). Another approach to antibody purification that is very popular in recent times is the use of specific immunosorbents. The use of antigens covalently coupled to an insoluble carrier, for example the diazotization product of p-aminobenzyl cellulose, for purification of antibodies was introduced by Campbell et al (21). Givas et al have used a myoglobinethylenemaleic anhydride copolymer for the purification of antimyoglobin antibodies (22). Antigen-bromoacetylcellulose conjugates have been used extensively for the purification of antibodies

to natural proteins, haptens and synthetic polypeptides (23). The advantages of the specific purification procedures are that antibodies are recovered in very high yield and purity with little, if any, loss of their activity.

# The Structure of Immunoglobulins and Antibodies

Immunoglobulins are a large, heterogeneous population of serum proteins containing antibodies of different specificities. Five different classes of immunoglobulins can be defined according to their general properties and the class of their heavy chains [Table I] (24).

Immunoglobulins belonging to isotypic classes and subclasses are distinguishable by the structural properties of their heavy chains, the light chains ( $\kappa$  or $\lambda$ ) being common to all immunoglobulins. These properties are manifested in certain biological functions such as complement fixation, binding to tissue, antigenicity etc. and are probably unrelated to the binding site (25, 26). Genetically controlled variations in immunoglobulins may also occur among individuals of the same species (27). For reasons of brevity, the following discussion will be confined mainly to the properties of the rabbit IgG molecule.

Papain digestion of  $\gamma$ globulin antibody in the presence of cysteine gives rise to three fragments which can be separated by chromatography on carboxymethylcellulose. Two of these fragments, the Fab fragments, retain full combining capacity for antigen but are univalent. The third fragment, the Fc piece, has been shown to be completely devoid of affinity for antigen while retaining

### <u>Table I</u>

### Classes of Human Immunoglobulins

Class	γG	γA	үМ	γD	γE
Heavy Chains :					
class	γ	α	μ	δ	ε
subclasses	Y1 , Y2 , Y3 , Y4	α1,α2	<del></del>	<u>_</u>	
mol. wt.	53,000	64,000	70.000		75,000
Light Chains : (mol.wt. 22,500)	κ,λ	κ,λ	κ,λ	κ,λ	κ,λ
mol. formula	( $\kappa_2\gamma_2$ ) or ( $\lambda_2\gamma_2$ ) <sub>n</sub>	(κ <sub>2</sub> α <sub>2</sub> ) <sub>n</sub> or (λ <sub>2</sub> α <sub>2</sub> ) <sub>n</sub> n=1,2,3	$(\kappa_{2}\mu_{2})_{5}$ or $(\lambda_{2}\mu_{2})_{5}$	( $\kappa_2\delta_2$ ) or ( $\lambda_2\delta_2$ )	(κ <sub>2</sub> ε <sub>2</sub> ) or (λ <sub>2</sub> ε <sub>2</sub> )
<sup>S</sup> 20,w	6.5-7.0	7,10,13,15	18-20	6.2-6.8	7.9
Molecular Weight	150,000	180,000-500,000	890,000		196,000
Carbohydrate (%)	2.9	7.5	11.8		10.7

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the major antigenic determinants common to  $\gamma G$  globulins (28, 29). Pepsin in the absence of a reducing agent cleaves IgG into a divalent fragment,  $F(ab')_2$ , with a molecular weight of about 100,000; the Fc piece is degraded into a number of smaller peptides (30). The production of univalent fragments depends upon the splitting of a single, labile disulfide bond linking the Fab' fragments together (31). Resistance of Fab (or Fab') fragments to further enzymatic cleavage indicates that the fragments are compact units. Cahnmann et al have shown that rabbit IgG can be split chemically by cyanogen bromide in a manner entirely analogous to limited peptic digestion (30). The various positions of cleavage are illustrated in Fig. 2.

IgG immunoglobulins have been shown to contain two classes of polypeptide chains which can be separated by gel chromatography after reduction and alkylation of the interchain disulfide bridges. Because of extensive non-covalent interactions among the chains, their separation is usually brought about in urea (33), acid (34, 35) or in detergent (36, 37). Each IgG molecule consists of two heavy  $(\gamma)$  chains (mol. wt. of about 53,000) and two light ( $\kappa$  or  $\lambda$ ) chains (mol. wt. of about 22,500) (38) as represented in the schematic diagram, Fig. 2.

The number of interchain disulfide bonds in  $\gamma$ G immunoglobulins can be determined by amino acid analysis and sequencing. One disulfide bond connects the carboxy-terminal half-cysteine residue of the light chain to a half-cysteine residue in the heavy chain (39). The number of inter-heavy chain disulfide bonds reported varies between 1-3 among the subclasses of immunoglobulins. Nisonoff and Dixon

found that in rabbit IgG about 60 per cent of the IgG population could be dissociated into univalent half-molecules by reduction of a single disulfide bond (40). The remaining 40 per cent may possess a disulfide bond of less lability to reduction or may have more than one inter-heavy chain bond. This disulfide bond linking half-molecules of IgG has been identified as the same bond holding the univalent fragments together in the  $F(ab')_{2}$  dimer. The failure of Fab monomers to reoxidize into dimers implies that the interheavy chain disulfide bond must lie between the positions of cleavage by pepsin and papain (41). Antibodies reconstituted from halfmolecules of purified antiovalbumin antibody and normal IgG blocked the homologous precipitin reaction (42). Such hybrids are therefore univalent, in accord with the structure of one specific heavy chain and one specific light chain. Similar univalent hybrids have been reconstituted from the Fab' fragments obtained by pepsin digestion (31).

The light chains have a remarkable amount of internal symmetry. They are made up of two distinct regions of nearly equal length, each region containing a disulfide loop of about 60 amino acid residues. One half comprises the amino-terminal region of variable amino acid sequence, while the other, the carboxy-terminal region of constant amino acid sequence, is covalently linked to the heavy chain (43). The variable region differs markedly from one  $\kappa$  light chain to another and between  $\kappa$  and  $\lambda$  chains, while the invariant region has a unique sequence for each class of light chain and for each species (44). The carboxy-terminal half of the heavy chains of  $\gamma$ G immunoglobulins

## Figure 2

Schematic diagram of the IgG molecule. The positions of peptide bond cleavage produced by pepsin, papain and cyanogen bromide are shown.



is covalently folded in a fashion similar to the light chains, with a carboxy-terminal and an amino-terminal loop (45). The Fd fragment of the heavy chain also has a constant and variable region with two intrachain disulfide bridges (46).

The function of the variable and constant regions cannot be stated explicitly. However, the predominant supposition is that the variable regions of both chains are responsible for the diverse specificities of the antibodies as well as the heterogeneity of affinity for the same hapten, whereas the constant regions of the light chains and Fd fragments determine the interchain complementarity in their association to form the combining site (37, 47). The intrachain disulfide bonds seem to lock into place a stable configuration of the chains.

### Gross Features of Macromolecular Structure

### Overall Shape and Flexibility

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Noelken et al (48) have proposed a model for the IgG molecule consisting of three compact units, two Fab and one Fc fragments, connected by an extremely flexible polypeptide region. The degree of flexibility about these peptide linkages was investigated by studying the rotational relaxation times of fluorophores conjugated to the molecule. There is quite a disagreement in reported relaxation times, 220 nanoseconds (rigid model) (49, 50) as opposed to 100 nanoseconds (extremely flexible model) (51). The consensus of opinion is in favour of the model proposed by Noelken et al. This model is supported by electron micrographs of antigen-antibody (52) and divalent hapten-antibody (53) complexes: Y-shaped antibody

molecules were found linked by divalent hapten molecules, the angle between the Fab moleties varying from  $0^{\circ}$  to  $180^{\circ}$  with increase in size of the hapten-antibody complex (53). The dimensions of the Fab portions were found to be 60 Å x 35 Å and those of the Fc portion 45 Å x 40 Å. The molecular weights calculated from these dimensions agree with the observed values of 50 - 55,000 for the Fab and 48,000 for the Fc fragment (48).

Hydrodynamic studies have shown that the IgG molecule has a frictional coefficient ratio of 1.47, substantially higher than the range of values of 1.1 to 1.3 expected for a typically globular protein. Fab and Fc fragments have frictional coefficient ratios of 1.24 and 1.21 respectively, suggesting a more compact structure for the fragments than for the IgG molecule.

Optical rotatory dispersion measurements of IgG yield low values of the Moffit-Yang constant,  $b_0$ , suggesting the absence of  $\alpha$ -helix (54). Similar studies in the ultraviolet region, 220-250 mp, show the absence of pronounced Cotton effects common in proteins with substantial  $\alpha$ -helix (55). Steiner and Lowey were unable to detect any conformational differences between antibodies and inert  $\gamma$ Globulin from their optical rotations (56). In one instance, however, Fab fragments from antibodies specific to the 2,4-dinitrophenyl determinant were found to differ from non-specific Fab fragments in having more  $\beta$ -structure and less random coil conformations (57).

### Location of the Antibody-combining Site

As was mentioned previously, the antibody-combining region resides somewhere in the Fab fragment, made up of a light chain and the Fd region of the heavy chain. The question arises as to whether the binding site is associated solely with one chain, the other participating only indirectly, or do both chains contribute directly to the antibody-combining site.

Refined isolation techniques make it possible to obtain from specific antibody the light chain and heavy chain (or Fd piece), practically free from mutual contamination, for the purpose of studying their individual antibody activity. These techniques however, involve rather drastic experimental conditions, and a certain irreversible denaturation of structure ensues which must be taken into account when considering the relative affinity of the subunits with respect to that of the intact antibody.

The evidence in favour of heavy chain activity heavily outweighs that involving specific binding of hapten by light chains. Metzger and Singer (58) and Givol and Sela (59) reduced and alkylated antigen-antibody complexes and found on column chromatography that the hapten was associated with the heavy chain peak, the light chain fraction showing no activity. Utsumi and Karush (36) measured the affinity of heavy chain for hapten directly by equilibrium dialysis; the residual activity in the heavy chain, expressed in terms of affinity constants was about 15 per cent of that of the intact antibody, the loss having been attributed to denaturation by detergent during the isolation procedure. In the cases mentioned above there was as much as 15 mole per cent contamination by light

chains, so that the evidence presented is not conlusive.

Haber and Richards (60) applied equilibrium dialysis and fluorescence quenching techniques to measure quantitatively the affinity for hapten of the chains obtained from polyalanylated antibody specific to the 2,4-dinitrophenyl determinant. No light chain antibody activity was found; the affinity constant of the heavy chain-hapten interaction was about 2 orders of magnitude lower than that for the intact parent antibody's reaction with hapten. This antibody activity was shown to be confined to the Fd region of the heavy chain (61); there were 0.25 sites associated with the heavy chain.

Specific binding of hapten by light chains has also been reported, but the affinity constants were so small that they could be determined only by highly sensitive methods of assay. Yoo et al (62) found that the fluorescence of the fluorophore-hapten, 4-anilinonaphthalene-l-sulfonate, was markedly enhanced on mixing with specific light chains; the calculated affinity constant, Ko, was about 10<sup>-2</sup> litre. mole<sup>-1</sup>. The value of the affinity constant is questionable since it indicates spontaneity in dissociation of the hapten-light chain complex. Moreover, Parker and Osterland (63) using a similar fluorophore, 8-anilinonaphthalene-1-sulfonate, observed strong fluorescence enhancement by both heavy and light chains from antibodies specific to different haptens (dinitrobenzene and ovalbumin) and from normal  $\gamma$ Globulin. The paired-labeling technique was used by Roholt et al (64) to trace unevenly labeled peptides to the light chain combining site. It is possible though that tyrosine residues in the heavy chain binding site may not be as

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readily available for labeling, and also that the labeled residues may not be associated with the binding region but may have been exposed by hapten-induced conformational changes. To substantiate this argument, Metzger et al (65) used the affinity-labeling technique to show that specifically bound diazonium haptens will react with tyrosine residues on both heavy and light chains, implying that these tyrosine residues are at or near the active sites. Similar results were obtained with antibodies specific to two different haptens (66); a fixed ratio of 2:1 was found for the amount of label on the heavy to that on the light chain.

Another approach to the location of the active site consists of reconstituting the antibody molecule from heavy and light chains and to compare the relative contributions of the chains to the specificity and affinity of the product. Roholt and his coworkers (67, 68, 69) found that the activity of the heavy chain was strongly enhanced when recombined with specific light chains and to a lesser extent when recombined with non-specific light chains. Negligible activity was found in hybrids reconstituted from specific light chains and inactive heavy chains (60). Recombination of specific heavy chains with non-specific light chains produced the same recovery of antibody-combining sites as with specific light chains (about 50 per cent) although the average affinity constant was much lower in the former case  $(1.5 \times 10^7)$ compared to  $2.6 \times 10^8$  M<sup>-1</sup> ) (60). It seems therefore that the heavy chains are of primary importance in determining the structure of the combining site, whereas the light chains play an indirect

role probably by stabilizing the conformation of the combining site on the heavy chain.

#### Nature and Size of the Antibody-combining Site

Two general theories have been proposed to explain the structural basis of antibody specificity. One is that the antibody with a given amino acid sequence can be folded into different conformations, and that each conformation corresponds to a different specificity. The second view maintains that it is the amino acid sequence alone that dictates an antibody's specificity and conformation. Support for the latter view has been obtained from the studies of Haber (70) and Whitney and Tanford (71). Renatured antibody Fab fragments which had been completely reduced and unfolded, showed as much as 25 per cent recovery of antibody activity. Statistically such high recovery cannot be brought about by random formation of disulfide and non-covalent bonds. The directive information for the conformation of the binding site must therefore lie in the amino acid sequence.

Antibodies to charged haptens usually have a small increase in the proportion of one or more oppositely charged amino acids. For example, antibodies directed against the positively charged determinant, phenyl-trimethylammonium, have been found to contain a negative charge in the binding site identified with the carboxylate group of aspartic acid (72, 73). The combining sites of antibodies to the apolar determinants, 2,4-dinitrobenzene (74) and 5-dimethylaminonaphthalene-1-sulfonamide (75), have been shown to be essentially hydrophobic in character.

Consistent differences in amino acid composition have been found in antibodies of different specificities (37), but these differences would seem to be too few to account for the diverse specificities of antibodies. The structural basis of antibody specificity must therefore include amino acid composition and sequence, the three-dimensional folding of each chain, and the complementarity of fit of the light and heavy chains to form the antibody-combining site. The actual size of the site is quite small (25 to 30 Å x 10 Å x 6 Å) compared to the size of the Fab molety (76), but the "contact" amino acids directly involved in the binding of hapten may come from widely separated positions along the length of the chains, brought together by the folding of the chains. This may explain why the presence of hapten stabilizes the whole Fab moiety to unfolding agents (77).

### Combining Site Heterogeneity

Antibodies directed against a single determinant group vary in their affinity for a single hapten and also in their capacity to bind structurally related haptens (78). The heterogeneity may stem from the fact that the hapten forms different structural antigenic determinants when coupled to a complex carrier molecule. The heterogeneity of antibodies is also reflected in the heterogeneity of electrophoretic mobility of the light chains. Light chains from specific antibody when analyzed by disc electrophoresis in polyacrylamide gels in alkaline buffers showed 6 to 10 sharp bands whereas myeloma light chains showed only 1 to 2 bands (79). The heterogeneity of the antibody is controlled not only by the charge

of the haptenic determinant but also by the charge of the carrier molecule (80). The differences in electrophoretic mobility may be attributable to differences in amino acid composition and sequence (81). It might be expected that a more homogeneous population of antibodies would be produced with immunogens containing a single antigenic determinant. However, antibodies elicited by monosubstituted 2,4-dinitrophenyl-ribonuclease still showed a certain degree of heterogeneity (82), so that heterogeneity cannot be minimized until rigidity of structure of the immunogen is assured as well (83).

The degree of heterogeneity and the affinity of binding both vary with time after immunization (84). This variation in affinity has been attributed to the type of antibody produced by different cells; it is presumed that a single cell would produce homogeneous antibodies. Nussenzweig and Benacerraf (85) found a predominance  $\lambda$ -type light chains in early low-affinity guinea pig antibody of  $\kappa$ -type light chains in late high-affinity preparations. and The rate of transition from low-affinity to high-affinity antibodies varies in different rabbits (86), and it is possible that there are also individual differences in the rate at which there is a shift toward synthesis of molecules with a particular type of light chain. There may also be a transition in the synthesis of different types of heavy chains with the time after immunization. McGuigan and Eisen (87) found that late high-affinity anti-dinitrophenyl antibodies differed from early low-affinity antibodies in having four more tryptophan and four fewer alamine residues per molecule. These differences were located in the Fd piece of the heavy chain.

Differences in affinity must also be related to tertiary structure. High-affinity anti-trinitrophenyl antibodies and early low-affinity anti-dinitrophenyl antibodies had identical optical rotation spectra which were quite distinguishable from the spectrum of high-affinity anti-DNP antibodies (56).

### CHAPTER II

### EQUILIBRIUM AND KINETIC STUDIES OF ANTIGEN-ANTIBODY AND HAPTEN-ANTIBODY REACTIONS

#### EQUILIBRIUM STUDIES

The simplest mechanism which can be postulated for antigenantibody and hapten-antibody reactions can be represented by the expression

$$Ab + H \xrightarrow{k_{12}} AbH$$
 (1)

where Ab represents the antibody combining sites, H the hapten and  $k_{12}$  and  $k_{21}$  the forward and reverse rate constants respectively for the one-step mechanism.

This expression of homogeneous equilibrium does not hold in the case of antigen-antibody reactions, between a bivalent antibody molecule and a multivalent, multideterminant antigen molecule. Complications arising from aggregational phenomena and the multispecificity of the reaction make interpretation of the reaction mechanism exceedingly difficult. Early equilibrium studies on antigenantibody reactions were performed in the limited region of antigen excess by Singer and Campbell (88) with the aid of ultracentifugal and electrophoretic techniques. More recent thermodynamic investigations by Dandliker et al (89) involved tagging one of the reactants, the antigen ovalbumin, with a small fluorescent label; the extent of combination of ovalbumin with anti-ovalbumin antibody was followed by measuring the changes in polarization of fluorescence of the label. This technique, because of its very high sensitivity, makes it possible to work at very low-concentrations of protein. The unique potential of the fluorescence polarization method arises from the fact that only the primary combination between the antigen and antibody molecule is needed to produce the experimental effect.

In hapten-antibody reactions problems due to secondary interactions between antigen and antibody e.g. precipitation, are avoided, and the reactions may be studied with relative ease using several techniques.

#### (a) Equilibrium Dialysis

In this technique the hapten and antibody solutions are placed in two separate compartments of a cell, separated by a membrane which is impermeable to protein but which allows free passage of hapten. After equilibrium is reached, the amount of hapten bound by the antibody can be ascertained from the decrease in its concentration in the protein-free compartment. Hapten concentrations can be measured spectrophotometrically (90) or by measuring radioactivity\_if a radioactively labeled hapten is used (61).

### (b) Spectrophotometric Method

Since bound molecules are in a different environment from that of the aqueous solution, it is not surprising that spectra of molecules that can be bound are modified in the presence of suitable proteins. Chemical compounds that are especially suitable for studying hapten-antibody interactions are naphtholic azo dyes which show

a considerable colour change on ionization of the naphtholic OH group. The spectral changes produced on binding by antibody have been identified with a pK shift of the dye due to hydrophobic interactions between the hapten and amino acid residues in or near the antibody combining site (91). However, smaller spectral changes which could not be attributed to a pK shift of the dye-hapten were also observed (92). The spectral changes may be used to calculate the extent of binding (93). In an antibody solution which contains both bound and free hapten, the absorption of light may be expressed as the sum of the contributions of both forms of hapten:

$$0.D. = \varepsilon_{f} c_{f} + \varepsilon_{b} c_{b}$$
(2)

where  $\varepsilon_{f}$  and  $\varepsilon_{b}$  are the molar extinction coefficients of the free and bound forms of the dye respectively and  $c_{f}$  and  $c_{b}$  the corresponding concentrations. If the apparent extinction coefficient of the dye is defined by the relation

$$0.D. = \epsilon_{app}(c_f + c_b)$$
(3)

then it follows that

$$\alpha = \frac{\varepsilon_{app} - \varepsilon_{b}}{\varepsilon_{f} - \varepsilon_{b}}$$
(4)

where  $\alpha$  is the fraction of total hapten which exists in the free state. In practice  $\varepsilon_{f}$  and  $\varepsilon_{b}$  (in antibody excess) are determined independently, and  $\varepsilon_{app}$  is determined from spectrophotometric measurements on antibody solutions titrated with measured quantities of hapten solution.

An alternative method used in this study entails measuring optical densities, after each addition of the dye-hapten, at two wavelengths:  $\lambda_1$ , where  $\varepsilon_{f1} >> \varepsilon_{b1}$  and  $\lambda_2$ , where  $\varepsilon_{b2} >> \varepsilon_{f2}$ , and solving for  $C_f$  and  $C_b$  in the two simultaneous equations:

$$0.D. = \epsilon_{\mathbf{f}1} \mathbf{C}_{\mathbf{f}} + \epsilon_{\mathbf{b}1} \mathbf{C}_{\mathbf{b}}$$
(5)

$$0.D._{2} = \begin{array}{c} \varepsilon & c \\ f_{2} & f_{2} \\ f \\ \end{array} + \begin{array}{c} \varepsilon & c \\ b_{2} \\ b \\ \end{array}$$
(6)

This method is more precise than the previously mentioned one since it does not depend on the addition of accurately known amounts of dye-hapten. Moreover, with the proper selection of wavelengths, both free and bound forms of the dye can be measured with the same sensitivity. The precision of the spectrophotometric method depends on the magnitude of the difference in absorption produced by the presence of the protein. Frequently this difference is small and not adaptable to quantitative measurements.

### (c) Fluorometric Titrations

(i) <u>Fluorescence Quenching</u>. A much more sensitive assay of quantitative binding of hapten by antibody is the measurement of the quenching of protein fluorescence by the hapten. Fluorescence quenching occurs as a result of radiationless transfer of energy from an excited fluorescent species, the antibody molecule, to the recipient molecule, the hapten, through dipole-coupling. The conditions for energy transfer are

(a) that the two species are favourably orientated with respect to each other, and
(b) that the absorption spectrum of the recipient molecule

of the donor. These conditions are highly favourable in some haptenantibody systems. When a hapten molecule behaves as the recipient in energy transfer, it quenches the fluorescence of tryptophan residues in or near the antibody-combining site. The effect is additive so that the quenching of fluorescence can be used for the determination of the stoichiometry as well as the association constant of the reaction (94, 95). Highly purified specific antibody preparations must be used to prevent loss of sensitivity owing to the presence of fluorescent, inactive normal  $\gamma$ -globulin.

(ii) <u>Fluorescence Enhancement</u>. This technique is very similar to fluorescence quenching. In this method a fluorescent hapten is used which has a very low quantum yield in aqueous solution. On binding to antibody the hapten is transferred to a hydrophobic environment and is protected from solvent collisions. As a result its fluorescence is strongly enhanced. Like fluorescence quenching the effect is additive and can be used as a measure of the extent of binding (75, 96).

### Treatment of Binding Data

From the law of mass action, a relationship between the concentrations of free [c] and bound [b] hapten can be derived for the conditions: (a) that the equilibrium constant, K, for the combination of hapten with protein is the same for all the binding sites, and (b) that the combination of one hapten molecule with the antibody does not appreciably alter the affinity of the antibody for another hapten molecule. Then

$$\frac{1}{b} = \frac{1}{K[Ab]c} + \frac{1}{[Ab]}$$
(7)

where [Ab] represents the concentration of total antibody sites. Equation (7) requires a linear relationship between 1/b and 1/c. In actual practice such a relationship is not generally observed. The curvature of a plot of 1/b versus 1/c has been attributed to the heterogeneity of combining sites with respect to the free energy of interaction (97, 98). Nisonoff and Pressman (99) employed a binding equation proposed by Sips (100) for the adsorption of gases on a solid surface and which contains a parameter that reflects the heterogeneity of the antibody population. This equation may be written

$$\frac{1}{b} = \frac{1}{\left(K_{o}c\right)^{\alpha}[Ab]} + \frac{1}{[Ab]}$$
(8)

where  $K_0$  is an average intrinsic association constant, and  $\alpha$  is the index of heterogeneity. For a homogeneous group of sites  $\alpha = 1$ , and equation (8) reduces to the simple mass-law expression (7). Decreasing values of  $\alpha$  correspond to increasing degrees of heterogeneity of association constants. Equation (8) predicts a linear relationship between 1/b and 1/c<sup> $\alpha$ </sup>. The experimental suitability of equation (8) and the determination of  $\alpha$  and  $K_0$  can be facilitated by the use of the following equation easily derived from equation (8):

$$\log\left(\frac{\mathbf{r}}{1-\mathbf{r}}\right) = \alpha \log c + \alpha \log K_0 \tag{9}$$

where r is the ratio of moles of bound hapten to the concentration of total antibody-combining sites (r = b/[Ab]).

The various thermodynamical parameters are calculated according to the following equations:

$$\Delta F^{O} = - RT \ln K_{O}$$

$$\frac{d \ln K_0}{d(1/T)} = -\frac{\Delta H^0}{R}$$
$$\Delta F^0 = \Delta H^0 - T\Delta S^0$$

where R is the gas constant, T is the absolute temperature, and  $\Delta F^{O}$ ,  $\Delta H^{O}$  and  $\Delta S^{O}$  are the usual standard free energy, enthalpy and entropy of the reaction.

#### KINETIC STUDIES

The utilization of the methods of chemical kinetics for the elucidation of the mechanism of the hapten-antibody reaction has provided some very interesting observations. Early attempts to investigate the reaction rates were made with the aid of cathode-ray polarography (101). The results of this study showed that the reaction had reached completion within 1 to 2 seconds which was the time of mixing of the components. Lower limiting rate constants of  $1 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$  and  $1 \text{ sec}^{-1}$  were derived for the forward and reverse reaction, respectively. A similar lower limiting value for the forward rate constant was obtained by Sturtevant et al (102) from the results of stopped-flow kinetic experiments.

On the basis of the existing theories of diffusion controlled reactions (103), it can be shown that the maximum value for the rate constant of a bimolecular reaction involving molecules with dimensions similar to those of haptens and antibodies is of the order of  $10^9 \text{ M}^{-1} \text{ sec}^{-1}$ . Therefore, in order to study the rapid hapten-antibody reactions, methods different from conventional techniques for slow reactions have to be employed. These include:
#### (a) The Temperature-Jump Relaxation Method :

Developed by Eigen et al (104), this technique is generally applicable to rapid reactions involving a change in enthalpy and which occur within the range of one microsecond to one second. The reaction system at equilibrium is perturbed by a sudden jump in temperature (of about  $6^{\circ}$ C) which is achieved within 0.1-1 µsec by discharging a condenser through the solution of the reaction system. The rate of readjustment to the new equilibrium conditions at the higher temperature is then determined by following the change in an appropriate physical parameter which represents the concentration of one of the reactants. This technique has been applied to the study of the kinetics of reaction of antibody with the haptens, arsanilic acid (105), 2,4-dinitro-and p-nitro-benzene (92). The forward rate constants were found to vary within one order of magnitude i.e within the range of  $2 \times 10^7$  to  $2 \times 10^8$  M<sup>-1</sup> sec<sup>-1</sup>, while much greater variation was observed for the reverse rate constants.

#### (b) The Stopped-Flow Method

This technique was originally developed by Hartridge and Roughton (106) for reactions in solution. The reactants are placed into separate chambers and driven by plungers through a mixing jet into the reaction chamber; the dead-time of mixing has been significantly reduced in improved instruments to less than 2 milliseconds (107). This technique is applicable to hapten-antibody reactions only if very low concentrations of reactants are used so that the half-times of reaction are significantly greater than the dead-time of mixing. Thus the reaction of anti-DNP antibody with  $\varepsilon$ -DNP-lysine was studied by Day et al by measuring the change in tryptophan fluorescence of the antibody molecule as a function of time (95); the half-time of the reaction using micromolar concentrations of reactants was 20 msecs. The limiting rate constants reported by the authors were all of the order of  $10^8 \text{ M}^{-1} \text{ sec}^{-1}$ .

Very few studies of the speed of antigen-antibody associations have been made. Kinetic data were obtained by light scattering from measurements of the intensity changes in the light scattered as a function of time (108). Berson and Yalow (109) studied the kinetics of interaction of I<sup>131</sup> - labeled insulin with its specific antibodies using concentrations of reactants as low as  $10^{-9}$  M and calculated forward rate constants of the order of 10<sup>4</sup> to 10<sup>6</sup> M<sup>-1</sup> sec<sup>-1</sup>. Interpretations of the kinetic results of polyvalent antigen-divalent antibody reactions are difficult to make because of the fact that many different and complex reactions may occur simultaneously obscuring the measurement of the primary combination of antigen with antibody. For example, re-equilibration steps may occur among the complexes affecting the distribution of the aggregates with respect to their size (110); steric repulsion among the bulky antigen molecules may also affect the rate of binding of successive antigen molecules to the same antibody molecule. Dandliker and Levison (111) eliminated some of these problems by working at extremely low concentrations of reactants and applying initial rate studies to the reaction of fluorescein-labeled ovalbumin with anti-ovalbumin antibody; rate measurements were carried out by measuring the polarization of fluorescence of the label as a function of time. They were able to show that in the initial stages of combination the empirical rate law is

rate =  $k_{12}$ [Ab][Ag]

where [Ag] and [Ab] are the initial antigen and antibody concentrations.

#### Conclusions from Thermodynamic and Kinetic Data

Thermodynamic and kinetic data provide the only quantitative information about the strengths of hapten- or antigen- antibody bonds and the factors that contribute to them. An inspection of Table II indicates that the association constants for most hapten-antibody systems are of the order of 10<sup>5</sup> M<sup>-1</sup>. The only exceptions are reactions involving the 2,4-dinitrophenyllysyl (DNP) group and some antibodies elicited by protein-DNP conjugates, for which association constants of the order of  $10^7 - 10^8 \text{ M}^{-1}$  have been observed (84, 94). The particularly high constants observed for this system may be explained as follows: (i) High-affinity anti-DNP antibodies are usually elicited by small amounts of the immunogen in Freund's Adjuvant and are thus not removed by circulating immunogen. In contrast most of the other antibody preparations listed in Table II were produced by intravenous injections of comparatively large amounts of the immunogen. (ii) Antibodies elicited in response to hapten-protein conjugates are actually directed against immunogenic groups larger in size than the hapten used in physico-chemical studies i.e. amino acid residues in the vicinity of the covalently bound hapten are included in the antigenic determinant. Thus anti-DNP antibodies may also be directed against the lysine moiety of 2,4-dinitrophenyllysine. As would be expected anti-DNP antibodies bind 2,4-dinitroaniline less strongly than  $\varepsilon$  -DNP-lysine (see Table II). This reasoning may also explain the high affinity constant  $(10^9 \text{ M}^{-1})$  for the reaction between insulin and anti-insulin antibodies (109).

With the exception of the reactions of Lac-dye: anti-Lac and some DNP: anti-DNP systems, AS<sup>O</sup> is usually positive for haptenantibody and antigen-antibody reactions. The entropy contributions

Tab1	.e	II
<u> </u>	_	

Thermodynamic Parameters for hapten-antibody and antigen-antibody reactions

System	к <sub>о</sub> [м <sup>-1</sup> ]	-∆F <sup>0</sup> [kcal. mole <sup>-1</sup> ]	-∆H <sup>0</sup> [kcal. mole <sup>-1</sup> ]	∆S <sup>0</sup> [e.u.]	Reference
p-iodobenzoate : anti-p-azobenzoate antibodies	4.1 x $10^4$	6.3	4.1	7.3	(112)
p-(tyrosineazo)-benzene sulfonic acid : anti-p-azo benzene sulfonate antibodies	-	8.97	8.39	2.0	(113)
D-phenyl-[p-(p-dimethylaminobenzeneazo)- benzoyl-amino]-acetate (D-I <sub>p</sub> -dye) : anti-D-I <sub>p</sub> -dye antibodies	3.1 x 10 <sup>5</sup>	7.24	7.1	0.3	(114)
p-(p-dimethylaminobenzeneazo)-phenyl β-lactoside (Lac-dye) : anti-Lac-dye antibodies	1.6 x 10 <sup>5</sup>	7.09	9.7	8.8	(115)
2,4-dinitroaniline : anti-2,4- dinitrophenyllysyl antibodies	2.2 x 10 <sup>6</sup>	8.86	16	-26	(84)

System		К <sub>о</sub> гм <sup>-1</sup> 1	$-\Delta F^{o}$	$-\Delta H^{o}$	۵S <sup>o</sup>	Reference
	· · · · · · · · · · · · · · · · · · ·					
2,4-dinitrophenylysine : anti	7					
phenyllysyl antibodies	(i)	$2.3 \times 10^{-1}$	10.89	19.6	-30.4	(84)
	(ii)	$2.3 \times 10^{5}$	6.8	1.6	17	(116)
	(iii)	$2.0 \times 10^8$	11.3	8.6	4	(94)
Bovine serum albumin (BSA) : antibodies	anti-BSA	<del>-</del> .	5.5	0±2	19±8	(117)
Ovalbumin : anti-ovalbumin ar	ntibodies	$1.2 \times 10^4$	5.6	0±2	20±8	(88)
Multivalent BSA-azobenzenears anti-azo-benzenearsonate anti	sonate : lbodies	$3.1 \times 10^3$	4.8	0±1	18±4	(118)
Terephthalamide diarsonate : benzenearsonate antibodies	anti-azo-	$3 \times 10^5$	7.4	0.8±2.6	22±9	(119)

# Table II (continued)

# Thermodynamic Parameters for hapten-antibody and antigen-antibody reactions

to the  $\Delta F^{O}$  of reaction are generally much greater than the enthalpy contributions for bivalent hapten-antibody and polyvalent antigenantibody reactions [Table II, listed under (117, 88, 118 and 119)]. To account for the positive  $\Delta S^{O}$  Kauzmann (120) has suggested that the increase in entropy is due to hydrophobic bonding i.e. to the release of water of hydration associated with the apolar substituents of the hapten and the antibody-combining site when the complex is formed. This, however, does not explain the far greater  $\Delta S^{O}$ contributions in antigen-antibody reactions unless one assumes hydrophobic interactions, other than those involving the antigenic determinant and the antibody-combining site, are participating in the reactions. Even in the reaction of antibody with the apolar hapten, 5-dimethylaminonaphthalene-l-sulfonamide, which one would expect to be entropy driven, the major contribution to the free energy is still  $\Delta H^{O}$  (96). Karush (121) has offered the explanation that these large entropy increases may be the result of molecular rearrangements in the antibody molecule to a more flexible conformation.

Generalizations as to the relative enthalpic and entropic contributions to the free energy of hapten-antibody reactions are difficult to make. Inspection of the thermodynamic parameters listed for the three  $\varepsilon$ -DNP-lysine: anti-DNP antibody systems [Table II, (84, 94 and 116)] shows that the relative contributions of  $\Delta H^{\circ}$  and  $\Delta S^{\circ}$  to the free energy vary enormously. The major enthalpy contributions to hapten-antibody associations suggest that forces other than hydrophobic bonding are involved, most probably hydrogen bonding. These bonds in aqueous solution have little or no intrinsic stability (122) but may contribute significantly in non-polar or slightly polar media to the  $\Delta F^{\circ}$  of reaction. The micro-environment

33.

of the antibody-combining site may satisfy these conditions. The same may be said for electrostatic interactions between charged groups (123).

The results of rate measurements on hapten-antibody reactions indicate that the formation of the complex is quite rapid: the second order rate constants are in the range of  $10^7 - 10^8$  M<sup>-1</sup> sec<sup>-1</sup> [Table III]. The values of these rate constants are somewhat smaller than expected for a diffusion controlled process ( $10^9 M^{-1} sec^{-1}$ ) - this may be a reflection of the rigid steric requirements for binding. Rate constants of similar magnitude have been observed for reactions of enzymes with their substrates or with co-enzymes (124, 125). The speed of the reaction and the low activation energies argue strongly against any conformational changes taking place during the reaction; only a single relaxation effect has been observed (92, 95). The forward rate constants for antigen-antibody reactions, on the other hand, are much smaller  $(10^4 - 10^6 \text{ M}^{-1} \text{ sec}^{-1})$ , and, when considered in conjunction with the comparatively large activation energies (12 kcal. mole<sup>-1</sup>) and entropy changes for complex formation, might well be associated with a loosening of the antibody structure.

The dissociation rate constants vary considerably, and this variation is primarily responsible for the variation in binding constants. The values of  $k_{12}$  for the association of anti-DNP antibody with the dye-haptens, 1N-2,5S-4DNP and 1N-2,5S-4pNP, are about the same, and the five-fold difference in their equilibrium constants are reflected in their reverse rate constants -80 sec<sup>-1</sup> for 1N-2,5S-4DNP as compared to  $410 \text{ sec}^{-1}$  for 1N-2,5S-4pNP (92). Thus the binding constant of a hapten-antibody reaction appears

## <u>Table III</u>

# Rate Constants for hapten-antibody and antigen-antibody reactions at $25^{\circ}$ C.

Hapten or Antigen	<sup>k</sup> 12 [M <sup>-1</sup> sec <sup>-1</sup> ]	<sup>k</sup> 21 [sec <sup>-1</sup> ]	Reference
Insulin <sup>a</sup>	$\sim 10^4 - 10^6$	$10^{-5} - 10^{-3}$	(109)
Ovalbumin	$2 \times 10^5$	10 <sup>-3</sup>	(111)
Bovine serum albumin	∿10 <sup>5</sup>	∿10 <sup>−5</sup>	(126)
2-(2,4-dinitrophenylazo)-1-naphthol- 3,6-disulfonic acid	8 x 10 <sup>7</sup>	1.4	(95)
ε-N-2,4-dinitrophenyllysine	$8 \times 10^7$	1	(95)
1-hydroxy-4-(2,4-dinitrophenylazo)-2,5- naphthalene disulfonic acid (1N-2,5S-4DNP)	1.6 x 10 <sup>7</sup>	80	(92)
l-hydroxy-4-(4-nitrophenylazo)-2,5- naphthalene disulfonic acid (1N-2,5S-4pNP)	1.4 x 10 <sup>7</sup>	410	(92)
4,5-dihydroxy-3-(p-nitrophenylazo)-2,7- naphthalene disulfonic acid	1.8 x 10 <sup>8</sup>	760	(127)
p-(p-dimethylaminophenylazo)-benzene arsonic acid	$1.1 \times 10^{7}$	$1.4 \times 10^{-3}$	(128)

<sup>a</sup> These data were obtained at  $37^{\circ}C$ .

to be governed mainly by the "lifetime" of the complex and not by the rate at which the two reactants can combine. The heterogeneity of affinity of antibody for hapten seems to be due to a distribution of dissociation rate constants. Day et al (95) found single association and dissociation rate constants when working under conditions where the concentration of antibody sites was in appreciable excess, usually about two-fold, of the concentration of hapten. However, in experiments carried out with hapten concentrations in excess of antibody site concentrations, the kinetic data yielded plots of downward curvature, reflecting the kinetic heterogeneity of the system. This distribution of dissociation rate constants may also explain the large values for the equilibrium constant obtained by Froese (92) from kinetic experiments over those average values deduced by equilibrium studies.

#### SCOPE OF THIS STUDY

The aim of this investigation was to compare equilibrium and kinetic parameters for reactions of haptens and intact antibodies, on the one hand, with those of haptens and antibody subunits, on the other. The experimental section of this thesis is divided into two main parts:

(a) In the first section (Chapter III), a comparative study was made of the reactions involving univalent Fab' fragments, obtained from three different anti-DNP antibody preparations, with those of their corresponding bivalent parent antibodies. In one series of experiments, the affinity of antibody (or Fab' fragment) for the dye-hapten, 1-hydroxy-4-(2,4-dinitrophenylazo)-2,5-naphthalene disulfonate, was determined spectrophotometrically, and the corresponding reaction rate constants were determined using the temperature-jump relaxation technique. In a second series of experiments, equilibrium parameters for the reaction of  $\varepsilon$ -DNP-lysine with antibody or Fab' were determined using the fluorescence quenching technique, and rate measurements, as followed by fluorescence quenching, were carried out with the aid of the stopped-flow method.

(b) In the second section (Chapter IV), the residual antibody activity in the isolated polypeptide chains from specifically purified anti-DNP antibodies was measured by the fluorescence quenching technique, and kinetic studies, where applicable, were performed using the stopped-flow technique.

The results derived from these investigations are discussed in detail in the section entitled GENERAL DISCUSSION.

#### CHAPTER III

# KINETICS OF REACTION OF ANTIBODY AND Fab' FRAGMENTS WITH HAPTEN

#### INTRODUCTION

Ever since Nisonoff et al (29) had shown that little difference, if any, exists in the affinity for hapten between antibody and its univalent Fab' fragments, it has been assumed that there is no interaction between the Fab moieties of an antibody molecule. Later, McGuigan and Eisen (87) upheld this hypothesis, apparently attaching no significance to small differences in affinity constants when the binding of various dinitrophenyl haptens by anti-DNP antibodies was compared with that by their Fab' fragments. This assumption of independent combining sites seemed also to be validated by the results of kinetic studies on hapten-antibody reactions (92, 95) since the derived rate equations, based on this assumption, described the behaviour of the specific interaction quite nicely. Moreover, there was no evidence of conformational changes occurring in the antibody molecule when it combined with hapten. The only suggestion of interaction between the Fab moieties arose from the electronmicroscopic studies of antigen-antibody (52) and bivalent hapten-antibody complexes (53) that led the authors to postulate that the antibody molecule "clicked open" on reacting with hapten, the angle of opening between the Fab moieties varying with the size of the antigen.

The present study was undertaken to re-investigate the binding of hapten by Fab' fragments and, at the same time, to compare the

kinetic behaviour of these fragments with that of the intact parent antibodies. It was hoped that small differences in the binding constant for the reaction

$$Ab + H \xrightarrow{K_0^a} AbH$$
 (1)

might be amplified in the rate constants, since

$$K_0^a = \frac{k_{12}}{k_{21}}$$
,

and small differences in  $K_0^a$  might be brought about by larger, but unidirectional changes in the two rate constants.

The 2,4-dinitrophenyl (DNP) system was chosen since (i) some differences had been shown in the affinity for hapten between intact antibody and its univalent Fab' fragments, and (ii) this system is particularly suited for both equilibrium and kinetic studies.

#### EXPERIMENTAL

#### Preparation of Antigens

Bovine  $\gamma$ -globulin (Nutritional Biochemicals, Cleveland, Ohio), very nearly maximally substituted on  $\varepsilon$ -NH<sub>2</sub> groups of lysine residues by 2,4-dinitrophenyl groups, was used both to immunize rabbits and to precipitate antibodies from antisera. 2,4-dinitrophenylated human serum albumin (DNP-HSA) was used to prepare an antigen-cellulose immunosorbent for the specific purification of anti-DNP antibodies. These substituted proteins (DNP-ByG and DNP-HSA) were prepared by treating the protein solutions at alkaline pH with the sodium salt of 2,4-dinitrobenzenesulfonic acid (Eastman Chemicals) according to the procedure of Eisen et al (129). Limited substitution by DNP groups on HSA was accomplished by allowing the coupling reaction to proceed for 2 hours in the absence of excess DNP-sulfonate. Low molecular weight hapten conjugates were removed by chromatography on Sephadex G-25 after exhaustive dialysis against phosphate-buffered saline. The extent of substitution by dinitrophenyl groups was determined spectrophotometrically (129) from the ratio of absorbances at 360 and 280 mµ of the antigen solution in 0.01 M sodium hydroxide; the ByG was found to be substituted to the extent of 45 moles of DNP per mole of protein and HSA to the extent of 18 moles of DNP per mole of protein.

#### Immunization Procedure

Anti-DNP antibodies were obtained from rabbits which had been immunized with DNP-ByG. About 10 mg of the antigen in complete Freund's Adjuvant (Difco Laboratories, Detroit, Michigan) were injected subcutaneously in the abdominal region of the animals. After 2 weeks, the rabbits received weekly booster injections of 10 mg of antigen in complete Freund's Adjuvant, until sufficiently high-titered sera were obtained. The animals were bled 7 days after the last injection and again 3-4 days later. After a rest period of 2 months, they received a booster injection of 10 mg of antigen and after 7 days they were again bled twice within 5-7 days. The antisera were Seitz-filtered into sterile vials and stored in the frozen state.

## Purification of Antibodies

(a) <u>Non-specific</u>: The antibody preparation, Ab-1, was obtained from a pool of bleedings from a single rabbit. The  $\gamma$ -globulin fraction was precipitated with sodium sulfate (15).

(b) <u>Specific</u>: The antibody preparation, Ab-2, was obtained from a pool of several bleedings from <sup>4</sup> rabbits. The serum pool was incubated with ByG to remove any anti-ByG antibodies and then specifically

purified according to the procedure of Eisen and Siskind (84). The antibody preparation, Ab-3, was obtained from a pool of several bleedings from 5 rabbits. The antibody was isolated from the pooled serum with the aid of the specific immunosorbent, DNP-HSA-bromoacetylcellulose (DNP-HSA-BAC). This immunosorbent was prepared according to the method of Robbins et al (23); Whatman CC41 microgranular cellulose powder was used for the preparation of the bromoacetylcellulose. Antibodies were eluted from the immunosorbent using a 0.1 M solution of 2,4-dinitrophenol (E. Merck, Darmstadt, Germany) at pH 8.0; the dinitrophenol was removed by passage through an anion exchange column, Dowex 1 (x8, 200 to 400 mesh) in chloride form at neutral pH.

#### Preparation of Fab' Fragments

Univalent Fab' fragments were prepared from the three antibody preparations according to Nisonoff et al (130). The antibody in acetate buffer (pH 5.0) was hydrolysed with pepsin (Worthington Biochem. Corp., Freehold, N.J.) — 1.5% by weight of total protein — in the presence of 0.01 M L-Cysteine (Sigma Chem. Co., St. Louis, Mo.) for 18 hrs. at  $37^{\circ}$ C with stirring. At the end of the reaction, the mixture was adjusted to pH 8 and dialysed against Tris-HCl buffer (pH 8.0,  $\Gamma/2=0.1$ ). The Fab' digest was then passed through a column of Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, Calif.) to remove partially hydrolysed Fc fragments followed by chromatography on Bio-Gel P-200 to remove undigested  $\gamma$ -globulin and  $F(ab')_2$  dimers. The purity of the Fab' preparations was checked by centrifugation in the Beckman Model E analytical centrifuge.

#### Precipitin Analyses

The concentration of anti-DNP antibodies was measured by precipitin analyses according to Eisen (131) using DNP-HSA as the precipitating antigen. Antigen-antibody complexes were incubated for 1 hr at  $37^{\circ}$ C and then 24 - 48 hrs at  $4^{\circ}$ C. The amount of antibody in each washed precipitate, dissolved in 0.01 M NaOH, was calculated from the total absorbance at 280 mµ minus the absorbance attributable to antigen; the latter was calculated from the ratio of the extinction coefficients of DNP-HSA in 0.01 M NaOH at 280 and 360 mµ. The purity of the antibody preparations, Ab-2 and Ab-3, was evaluated by determining their precipitability by the DNP-HSA antigen.

The molecular weights used in all subsequent experiments were 150,000 and 50,000 for intact antibody and Fab' fragments, respectively. The corresponding extinction coefficients at 280 m<sub>µ</sub> ( $E_{lcm}^{1\%}$ ) used, were 15 and 15.9, respectively; the value of 15.9 was obtained by assuming  $E_{lcm}^{1\%}$  = 15 for  $\gamma$ -globulin (132) and using the ratio of extinction coefficients for Fab' and whole antibody (16.9: 15.9) reported by McGuigan and Eisen (87).

#### Haptens

The dye-hapten, 1-hydroxy-4-(2,4-dinitrophenylazo)-2,5-naphthalene disulfonate (1N-2,5S-4DNP), was prepared as previously described (92). Chromatographically pure  $\varepsilon$ -N-2,4-Dinitrophenyllysine hydrochloride ( $\varepsilon$ -DNP-lysine), was purchased from Mann Research Laboratories (Orangeburg, New York) and used without further purification; concentrations were determined from  $E_{360m\mu}^{1cm} = 1.74 \times 10^4 M^{-1}$  (95).

#### Equilibrium Studies

(a) <u>Spectrophotometric Method</u> The extinction coefficients at 470 and  $520_{m\mu}$  of the dye-hapten, 1N-2,5S-4DNP, bound to antibody were determined according to the method of Metzger et al (91) by adding increasing amounts of the dye-hapten to a large excess of antibodies or Fab' fragments; the extinction coefficient was calculated from the linear portion of a plot of optical density against hapten concentration.

Equilibrium studies of the binding of 1N-2,5S-4DNP by the antibody preparations, Ab-1 and Ab-3, and their univalent fragments were performed at  $25^{\circ}C$  in phosphate buffer (pH 6.0,  $\Gamma/2 = 0.1$ ). Small increments of the dye were added to 1 ml of protein ( $\sim 10^{-5}M$ ) and, after stirring, the optical densities were measured at 470 and 520mµ against the protein solution as a blank. All measurements were made in a Zeiss PMQ II spectrophotometer. The concentrations of bound (b) and free (c) hapten were calculated using the relationships:

$$b = \frac{\varepsilon_{f470}^{0.0.520} - \varepsilon_{f520}^{0.0.470}}{\varepsilon_{f470}^{0.520} - \varepsilon_{f520}^{0.0.470}}$$
(10)

$$c = \frac{\varepsilon_{b520}^{0.D.} 470 - \varepsilon_{b} 470^{0.D.} 520}{\varepsilon_{f} 470 \varepsilon_{b} 520 - \varepsilon_{f} 520 \varepsilon_{b} 470}$$
(11)

where  $\varepsilon_{f470}$ ,  $\varepsilon_{f520}$  and  $\varepsilon_{b470}$ ,  $\varepsilon_{b520}$  are the extinction coefficients of free and bound hapten, respectively, at the wavelengths indicated. 1/b was plotted against 1/c, and the binding curve thus obtained was extrapolated to 1/c =0 to yield the reciprocal of the total concentration of antibody sites,  $[Ab]_T$ , in solution. The average intrinsic association constant,  $K_o^a$ , was obtained by interpolation on the binding curve; according to equation (8),  $K_o^a = 1/c$  when  $1/b = 2/[Ab]_T$  i.e. when 50 per cent of the sites are occupied by hapten.

#### (b) <u>Fluorometric Titrations</u>

The binding of the hapten,  $\epsilon$ -DNP-lysine, by the purified antibody preparation, Ab-3, and its univalent fragments, Ab-3-Fab', was measured by the method of fluorescence quenching in an Aminco-Bowman spectrophotofluorometer according to Velick et al (94). All antibody and hapten solutions were prepared in 0.15 M NaCl, 0.01 M phosphate buffer, pH 7.4 — only double-distilled water was used to prepare the buffer, and all glassware used for handling the solutions in fluorescence studies were cleaned in concentrated nitric acid. The antibody and hapten solutions were passed through a millipore filter (0.45µ porosity) just prior to the titration experiments to remove any insoluble material.

Antibody samples (2 ml) in the concentration range of 40-120  $\mu$ g/ml were accurately transferred to a 1 cm-square quartz cuvette contained in a chamber maintained at 25°C. Fluorescence of the protein was excited at  $290_{m\mu}$  . After temperature equilibration (about 10 minutes), the initial fluorescence level at 350mu was recorded. Small aliquots (0.002 - 0.005 ml) of the hapten solution were then added by means of an "Agla" micrometer syringe. After each addition of hapten, the solution was stirred by means of a platinum coil (which remained in the solution during the titration), and the fluorescence level at 350mµ recorded after a fixed interval of 2-3 minutes for temperature equilibration. All titrations were carried out in duplicate. Control titrations of inert rabbit IgG were carried out to correct for nonspecific collision quenching and attenuation of incident and emergent radiation. In order to determine the maximum quenching of fluorescence, Q max, the antibody preparation was titrated

with a high concentration  $(5 \times 10^{-4} \text{ M})$  of  $\varepsilon$ -DNP-lysine to a final concentration of about 50 - fold excess over antibody site concentration without introducing a large dilution correction; in other titrations concentrations of 1 -  $2 \times 10^{-4}$  M  $\varepsilon$ -DNP-lysine in saline-phosphate buffer were used.

The emitted fluorescence intensity observed was corrected for solvent blank, dilution and normalized to 100 per cent initial fluorescence intensity. Corrections were then made for attenuation by hapten. The extent of attenuation at a particular concentration of ligand was dependent on the degree of overlap of the antibody fluorescence spectrum by the absorption spectrum of the ligand, and on the extinction coefficient of the ligand at the wavelength at which the fluorescence intensity was recorded. This hapten attenuation became quite significant in  $Q_{\max}$  determinations; at high concentrations of hapten the fluorescence intensity (or per cent transmission) was no longer linearly dependent on hapten concentration. To correct for the hapten attenuation, therefore, in contrast to standard methods (94), the logarithm of the fluorescence intensity was plotted against hapten concentration, and the terminal slope in the region of hapten excess was extrapolated to zero concentration of hapten. This method yielded Qmax values when 100 per cent of the antibody-combining sites were occupied by hapten.

After all corrections had been made, a specific quenching curve due to complex formation was obtained by plotting the corrected relative fluorescence intensity (RFI) as a function of hapten concentration. The concentration of antibody sites,  $(Ab)_{T}$ , in the sample at equivalence was obtained from the intersection of the

limiting slope of the fluorescence quenching curve at low hapten concentration and the horizontal line drawn at the level of  $Q_{max}$ . The fraction of sites occupied, r, at a particular concentration of hapten, (H)<sub> $\pi$ </sub>, was obtained from the relationship:

$$r = Q/Q_{\text{max}}$$
(12)

where Q is the observed quenching produced by bound hapten. The concentration, c, of free hapten was calculated according to the expression:

$$\mathbf{c} = (\mathbf{H})_{\mathbf{m}} - \mathbf{r}(\mathbf{Ab})_{\mathbf{m}} \tag{13}$$

The binding data were analyzed according to the Sips equation in its logarithmic form as suggested by Karush (133):

$$\log(\frac{r}{1-r}) = \alpha \log c + \alpha \log K_0^{a}$$
 (9)

The index of heterogeneity,  $\alpha$ , and the average intrinsic association constant,  $K_0^{a}$ , were determined from plots of  $\log(\frac{r}{1-r})$  against log c.

#### Kinetic Experiments

#### (a) Temperature-Jump Relaxation

The kinetic experiments with the dye-hapten, 1N-25S-4DNP, and the antibody preparations, Ab-1 and Ab-1-Fab', were carried out in phosphate buffer (pH 6.0,  $\Gamma/2$  =0.1) with a temperature-jump relaxation apparatus designed by L. de Maeyer and built in the workshops of the Max-Planck Institut für Physikalische Chemie, Göttingen, Germany. The temperature-jump method is particularly suited for reactions which occur in the time range of 1 µsec - 1 sec (104, 134) and yields both rate constants for the reaction represented by the equation

$$Ab + H \xrightarrow{k_{12}} AbH$$
 (1)

according to the relationship:

$$1/_{\tau} = k_{21} + k_{12}[(\overline{Ab}) + (\overline{H})]$$
 (14)

where  $\tau$  is the relaxation time, and  $(\overline{Ab})$  and  $(\overline{H})$  are the equilibrium concentrations of antibody-combining sites and hapten, respectively. The concentrations of bound and free dye-hapten were determined spectrophotometrically as described under 'equilibrium studies', page 43. All temperature-jump experiments were performed at 25°C at a wavelength of 520 mµ. To determine the relaxation time for a certain equilibrium concentration of antibody and hapten, results from 17 relaxation curves were averaged. The rate constants according to equation (14) were obtained by "least squares" analysis of all relaxation data.<sup>\*</sup>

#### The Temperature-Jump Apparatus

A block diagram of this apparatus is shown in Fig. 3. The high voltage power supply (0-30 KV) was connected through a 400 mega-ohm resistor to a 0.1 µF capacitor, which in turn was connected to the reaction cell (Fig. 4) through a variable spark gap. The cell was built of sturdy plexiglass to withstand the high pressures (30-50 atmospheres) generated by the discharged voltage. The platinum electrodes were separated by a distance of 11 mm. Two quartz rods were cemented into the "waist" of the cell for spectrophotometric observations. The total volume of the cell was about 15 ml, while the volume actually encompassed by the electrodes and the quartz windows was about 1 ml. The cell was surrounded by an insulated jacket, kept at constant temperature. The whole cell assembly was contained in a light-tight housing.

The derivation of the rate equation (14) and statistical treatment of the kinetic data for a typical experiment are given in Appendix A.

Figure 3

Schematic diagram of the Temperature-Jump Apparatus

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# Figure 4

Schematic diagram of the Temperature-Jump Cell

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. 49 The light source consisted of a 45 W quartz-iodide lamp, powered by five 12-volt auto batteries in parallel. After passing through an interference filter, the light beam was split by a halfsilvered mirror; one beam, the reference beam, passed through the air, while the other passed through the cell. The intensities of the two beams were measured by two photomultipliers (RCA IP28) powered by a stabilized power supply. The signal from the two photomultipliers was amplfied by a Tetronix plug-in unit (Type 53/54 D) and projected on a Tetronix oscilloscope screen.

The sequence of operation was as follows: The cell containing the solution to be studied was placed in its chamber and allowed to attain the pre-set temperature  $(19^{\circ}C)$  of the thermostat. The dark currents of the two photomultiplier units were balanced against each other. The light source was turned on, and the intensities of the main and reference beams were balanced against each other by means of iris diaphragms. The condenser was then charged slowly through the 400 mega-ohm resistor to a voltage of 30 KV. At this point, the condenser discharged through the spark gap, raising the temperature of the solution between the electrodes by  $6^{\circ}C$  within approximately 5 µsecs, simultaneously triggering the horizontal sweep of the oscilloscope.

The change of the intensity of the light beam passing through the reaction mixture was followed as a function of time. The relaxation pattern was photographed immediately. The heat generated in the reaction zone, 1 ml volume, was dissipated by convection into the bulk of the solution. Re-equilibration to the initial temperature required about 2 minutes. After this time a new discharge could be triggered.

#### (b) <u>Stopped-Flow</u>

Studies of the rate of quenching of fluorescence of the specifically purified antibody preparations, Ab-3 and Ab-3-Fab', by the hapten,  $\epsilon$ -DNP-lysine, were carried out in 0.15 M saline, 0.01 M phosphate buffer (pH 7.4) at 25<sup>o</sup>C with the aid of a Durrum-Gibson stopped-flow spectrophotometer (Durrum Instrument Corp., Palo Alto, Calif.). The solutions were excited at 280 mµ, and the emitted fluorescence radiation with wavelength longer than 295mµ was measured.

A general outline of the stopped-flow apparatus is given in Fig. 5. The spectrophotometer comprised five functional systems: the sample flow system, the flow actuating system, the optical system, the electronic system and the temperature control system.

The reaction components were stored in two large reservoir syringes (of 20 ml capacity) which in turn were connected through valves to the 2 ml drive syringes. The valve block, connecting the syringes and the Kel-F mixing jet outlet, was made of stainless steel. The cylindrical quartz reaction cuvette (2x20 mm) was arranged in such a way that the flow of liquid was along the axis of the incident light path when the instrument was used for absorption measurements. The cuvette was bounded at its extreme ends by quartz windows. In addition a plane mirror was angled above the length of the cuvette to reflect excitation radiation through the solution at right angles to the direction of flow. The outlet from the cuvette was connected through a drain valve block, made of stainless steel, to the "stop" To the plunger of this syringe an adjustable external stop syringe. was attached. This "stop" not only halted the flow after a certain predetermined volume had entered the syringe, but also actuated the

Figure 5

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Schematic diagram of the Stopped-Flow Apparatus

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trigger switch for the oscilloscope. A valve-controlled drain port was also connected to the drain valve block for removal of the reacted solution.

The original light source, a hydrogen lamp, provided with the instrument did not have sufficient intensity for fluorescence measurements. It was replaced by an Osram HBO 100 W/2 mercury high-pressure arc lamp, housed in a Zeiss microscope illuminator and powered by five 12-volt auto batteries in series. The lamp was started with an Osram Z 4000 ignition device. The light, after passing through a prism monochromator, could enter the reaction cuvette in one of two ways as regulated by two mirrors: parallel to the direction of flow of the reaction mixture or at right angles to it for fluorescence measurements. When the instrument was used for fluorescence measurements, an interference filter was placed in front of the exit window to prevent any stray excitation radiation from falling onto the photomultiplier. For the kinetic experiments described in this thesis, a filter was used which had 1% transmittance at 295mu and 70% transmittance at  $325 \text{m}\mu$ . The photomultiplier (EMI, type 9558) was powered by a Kepco constant voltage DC power supply with a continuously adjustable output voltage range of 0 - 1500 volts. The photomultiplier output signal, which was directly proportional to the light intensity, was amplified by a Tetronix plug-in unit, type 2A63, and displayed on a Tetronix type RM 564 storage oscilloscope.

The solutions in the mixing chamber — cuvette, valve block and drive syringes — were maintained at constant temperature in a closed circuit, open reservoir, circulating bath controlled by a Lauda K2-D thermostat.

An actual experiment was performed as follows: The flow system was flushed with nitric acid and rinsed thoroughly with double-distilled water and finally with the saline-phosphate buffer in which the reactants were dissolved. The solutions of the reactants were degassed and transferred to their respective reservoir syringes. Before filling the drive syringes with the separate solutions, each reactant solution was pushed back and forth between its drive and reservoir syringes to dislodge any trapped air bubbles. After allowing sufficient time for the solutions to reach the temperature of the thermostat bath (25°C), the flow actuator handle was struck sharply forcing equal volumes (0.3 ml) of the reactants through the flow system to the "stop" syringe; the minimum volume of each reactant required to completely displace the already reacted mixture in the reaction cuvette was 0.2 ml. Once good reproducibility was obtained, as observed on the oscilloscope screen, several determinations at different sweep rates were recorded photographically.

#### Treatment of Kinetic Data

Each reaction curve was smoothed by hand, and the extent of reaction was measured at various time intervals. The data were treated according to Day et al (95). Rate constants for the reaction represented by equation (1) were obtained by using the relationship:

$$\log \left(\frac{1-\phi_x}{1-x}\right) = \frac{k_{12}\sqrt{-Q}}{2.303} t$$
 (15)

See Appendix B for the derivation of rate equation (15).

$$\begin{split} \emptyset &= \left[ \frac{a_{0} + b_{0} + K_{0}^{d} - \sqrt{-Q}}{a_{0} + b_{0} + K_{0}^{d} + \sqrt{-Q}} \right], \\ \sqrt{-Q} &= \left[ (b_{0} - a_{0} + K_{0}^{d})^{2} + 4a_{0}K_{0}^{d} \right]^{\frac{1}{2}}; \ b_{0} > a_{0}, \\ a_{0} &= \text{ initial binding site concentration,} \\ b_{0} &= \text{ initial hapten concentration,} \\ K_{0}^{d} &= 1/K_{0}^{a}, \\ x &= \text{ fractional extent of reaction toward equilibrium at time, t; } x = 0 \text{ at } t = 0 \text{ and} \end{split}$$

x = 1 at  $t = \infty$ .

Log  $[(1 - \phi_x)/(1 - x)]$  values with time, t, were averaged from at least eight reaction curves and plotted by the "least squares" method. The second order rate constant,  $k_{12}$ , was calculated from the slope according to equation (15) and the reverse rate constant,  $k_{21}$ , was determined from the relationship:

$$k_{21} = k_{12}/K_{0}^{a}$$

It should be pointed out that, in order to analyze the kinetic data as described above, one had to determine the initial fluorescence level of the reaction mixture at zero time of reaction. According to standard procedure (95), this was effected by simply mixing the antibody solution with buffer in the stopped-flow apparatus; the fluorescence level would be practically the same as that for the antibody-hapten mixture at zero time of reaction, since the attenuation by hapten under experimental conditions was negligible. The initial slope of the reaction-produced fluorescence dropoff would then be extrapolated to

# Figure 6

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Analysis of a reaction waveform

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the initial fluorescence level (see Fig. 6). The time, on the horizontal time base axis, from this initial level to the beginning of the observed fluorescence dropoff (the system dead time) would correspond to the average age of the mixed solution when it came to rest in the reaction cuvette.

However, small changes in the fluorescence intensity and inherent instabilities in the mercury high-pressure lamp made it impossible to obtain fluorescence levels of the mixed reactants at zero time which could be related to fluorescence levels during the Therefore, recourse was taken to a system consisting of reaction. bovine serum albumin (BSA) and the dye, 1N-2,5S-4DNP, in phosphate buffer (pH 6.0,  $\Gamma/2 = 0.1$ ); the absorbance of the dye in buffer at  $470 \text{ }_{m\mu}$  was reduced markedly in the presence of BSA. These experiments could be performed with the rather stable tungsten-iodide lamp, under flow conditions identical to those used in fluorescence runs. Thus, this system was used to measure the instrument dead time, as described in the preceding paragraph, and which dead time could be used to determine the initial fluorescence level at zero time of reaction of antibody with  $\varepsilon$ -DNP-lysine.

#### RESULTS AND DISCUSSION

#### Antibody Purity

The precipitability of specifically purified antibody preparations, Ab-2 and Ab-3, was examined by the quantitative precipitin reaction according to the method of Farah et al (19). The preparations (1 mg/ml) were found to be 70 per cent precipitable with DNP-HSA antigen. 30 per cent of the antibody-combining sites

appeared to be occupied by residual antigen (or strongly bound  $\epsilon$ -DNP-lysine in preparation Ab-2). This suggestion was based on the similarity of the absorption spectra of purified antibody, DNP-ByG antigen and  $\epsilon$ -DNP-lysine in the wavelength range of 320 - 450mµ; in addition, the absorbance at 360mµ of a purified antibody solution of known concentration agreed closely with 30 per cent contamination of total binding sites by  $\epsilon$ -DNP-lysyl groups. Farah et al (19) had found that their anti-DNP antibody preparations were contaminated to the extent of 10 per cent of the antibody-combining sites. Chromatography of the contaminated antibody preparations on Bio-Gel P-300 or through an anion-exchange resin, Dowex 1(x8), failed to remove the antibody-hapten (or -antigen) complexes.

#### Isolation of Fab' Fragments

The elution pattern for the isolation of the Fab' pepsincysteine digest of the antibody preparation, Ab-3, is shown in Fig. 7. The major component from chromatography on Bio-Gel P-60 (Fig. 7A) was concentrated and rerun on Bio-Gel P-200 (Fig. 7B). The eluate under the major peak was collected as the Fab' component, dialysed against distilled water and lyophilized. The sedimentation pattern of Fab' fragments (10 mg/ml) in borate-saline buffer (pH 8.0,  $\Gamma/2 = 0.15$ ) is shown in Fig. 8. The calculated sedimentation coefficient was 3.7S (uncorrected).

## Figure 7B

Elution pattern of the major component shown in Fig. 7A rerun on a Bio-Gel P-200 column (4.5 x 120 cm) in Tris-HCl buffer (pH 8.0,  $\Gamma/2 = 0.1$ ) at room temperature.

### Figure 7A

Elution pattern of the pepsin-cysteine digest of antibody preparation Ab-3 from a Bio-Gel P-60 column (4.5 x 120 cm), equilibrated and developed in Tris-HCl buffer (pH 8.0,  $\Gamma/2 = 0.1$ ) at room temperature.




Sedimentation pattern of Fab' fragments (10 mg/ml) in borate-saline buffer (pH 8.0,  $\Gamma/2 = 0.15$ ) at 59,780 rpm (20<sup>o</sup>C). Photographs were taken after 48 minutes at 8 minute intervals.

### Antibody-Dye Interactions

The spectral properties and pH dependence of the dye-hapten, 1N-2,5S-4DNP, on binding by antibody has been reported by Froese (92). To prevent complications arising from the pH dependence of the reactions, all binding studies were performed at pH 6.0, under which conditions the bound and free forms of the dye exist in the protonated form only.

The effects of whole antibody, Ab-1, and its univalent fragments, Ab-1-Fab', on the spectrum of the dye-hapten are shown in Figs. 9 and 10, respectively. In the experiment illustrated in Fig. 9, since a large excess of antibody-combining sites was used, the two absorption peaks at 474 and  $497m\mu$  may be attributed to the free and bound forms of the hapten, respectively. Identical absorption spectra were observed for the bound dye-hapten, irrespective of whether the



Sedimentation pattern of Fab' fragments (10 mg/ml) in borate-saline buffer (pH 8.0,  $\Gamma/2 = 0.15$ ) at 59,780 rpm (20°C). Photographs were taken after 48 minutes at 8 minute intervals.

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The effect of anti-DNP preparation, Ab-1 (4 x  $10^{-4}$  M), on the spectrum of 1N-2,5S-4DNP in phosphate buffer (pH 6.0,  $\Gamma/2 = 0.1$ ). [A = free dye-hapten; B = bound dye-hapten].



, vtiznab Optical

The effect of various concentrations of Ab-1-Fab' on the spectrum of 1N-2,5S-4DNP (4.4 x  $10^{-6}$  M) in phosphate buffer (pH 6.0,  $\Gamma/2 = 0.1$ ).



hapten was bound by specifically purified antibodies (Ab-2),  $\gamma$ -globulin containing specific antibody (Ab-1) or their Fab' fragments. As is evident from Figs. 9 and 10, the absorption maximum was markedly shifted from 474 to 497mµ with a distinct lowering of the extinction coefficient of the dye-hapten. It should be pointed out that normal rabbit  $\gamma$ -globulin did not change the spectrum of the hapten.

The extinction coefficients of the bound hapten were determined at 470 and  $520m\mu$  (Fig.11), and are listed in Table IV, along with the extinction coefficients of the free hapten at these wavelengths.

#### Table IV

Extinction coefficients of lN-2,5S-4DNP in phosphate buffer (pH 6.0,  $\Gamma/2 = 0.1$ ).

		<u>470mu</u>	520mu
ε <sub>b</sub>	$[M^{-1} em^{-1}]$	$2.20 \times 10^{4}$	2.10 x $10^4$
بي 43	$[M^{-1} cm^{-1}]$	$3.68 \times 10^4$	$0.74 \times 10^{4}$

Practically identical values were obtained by Froese (92) for the extinction coefficients of 1N-2,5S-4DNP at 470m $\mu$  .

### Equilibrium Experiments

The extinction coefficients given above were used for the calculation of the extent of binding of the dye-hapten by antibody and by Fab' fragments according to equations (10) and (11). The data for the binding of 1N-2,5S-4DNP by antibody preparation, Ab-1, and by its univalent fragments, Ab-1-Fab' [Table V], were plotted as shown in Fig. 12, according to the method of Nisonoff and Pressman (99). Since, in the case of the Fab' preparation, Ab-1-Fab', the total

Determination of  $\varepsilon$  at 470 mµ (open circles) and 520 mµ (closed circles) for bound (b) 1N-2,5S-4DNP in Ab-1-Fab' (4 x 10<sup>-5</sup> M) at pH 6.0.

.



# <u>Table V</u>

Binding o	of 1N-2,5S-4DNP by Ab-1-Fab' (1.33 x 10 <sup>-5</sup> )	M)
	Spectrophotometric Method	

0.D.470mµ	0.D.520mµ	сх 10 <sup>5</sup> [М]	b x 10 <sup>5</sup> [M]	$1/c \times 10^{-5} [M^{-1}]$	1/b x 10 <sup>-5</sup> [M <sup>-1</sup> ]
0.142	0.099	0.132	0.425	7.60	2.35
0.208	0.135	0.229	0.563	4.37	1.78
0.292	0.166	0.406	0.648	2.46	1.54
0.375	0.192	0.598	0.705	1.67	1.42
0.468	0.219	0.820	0.755	1.22	1.32
0.556	0.244	1.03	0.800	0.97	1.25
0.643	0.264	1.26	0.816	0.79	1.23
0.731	0.285	1.49	0.836	0.67	1.20
0.847	0.314	1.78	0.871	0.56	1.15
0.963	0.342	2.08	0.900	0.48	1.11
1.075	0.367	2.37	0.915	0.42	1.09
1.23	0.402	2.76	0.946	0.36	1.06
1.37	0.435	3.13	0.973	0.32	1.03
1.51	0.469	3.50	1.01	0.29	0.99

Binding of 1N-2,5S-4DNP by Ab-1 (0.65 x  $10^{-5}$  M, closed circles) and by Ab-1-Fab' (1.33 x  $10^{-5}$  M, open circles) at 25°C in phosphate buffer (pH 6.0,  $\Gamma/2 = 0.1$ ).



number of antibody-combining sites in a given stock solution could only be determined from the binding data, binding studies were performed on three different dilutions of that stock solution of fragments. All three binding curves (Fig. 13) yielded the same number of antibody-combining sites per given volume of the original stock solution. Moreover, almost identical values were obtained for the average intrinsic association constant  $(K_0^a)$  as shown in Table VI.

#### Table VI

Estimation of Antibody Site Concentration in a stock solution of Ab-1-Fab' (see Fig. 13).

Dilution Factor	$1/[Fab']_T \times 10^{-5}$	[Fab'] <sub>T</sub> x 10 <sup>5</sup>	$[Fab]_{stock} \ge 10^5$	$K_0^{a} \ge 10^{-5}$
	[M <sup>-1</sup> ]	[M]	[M]	[M <sup>-1</sup> ]
l in 3	0.75	1.33	3.99	2.2
l in 4	1.00	1.00	4.00	2.3
l in 5	1.23	0.81	4.05	2.4

Average intrinsic association constants  $(K_0^a)$  were also determined for the antibody preparation, Ab-2, and its univalent fragments. The equilibrium constants obtained are within the range  $(\sim 10^5 \text{ M}^{-1})$ usually found for hapten-antibody reactions (Table II, Chapter II). Heterogeneity of binding of 1N-2,5S-4DNP was found for both anti-DNP antibody preparations and for their univalent fragments, as indicated by the curvature of plots of 1/b against 1/c (Fig. 12). It should be

Determination of the concentration of binding sites in a stock solution of Ab-1-Fab' in phosphate buffer (pH 6.0,  $\Gamma/2 = 0.1$ ) from binding data. Extrapolation of the plots to 1/c = 0 yields the reciprocal of the binding site concentration, [Fab']<sub>T</sub>.



pointed out that in both antibody preparations, the binding constants for Fab' fragments were somewhat higher than those for the corresponding intact antibodies. The results of all equilibrium studies are summarized in Table IX, page 76.

#### Fluorescence Quenching Studies

Binding of the hapten,  $\varepsilon$  -DNP-lysine, by anti-DNP antibodies was studied with antibody preparation, Ab-3, and its Fab' fragments, Ab-3-Fab'. As pointed out under "Experimental", it was necessary to determine  $Q_{max}$  values in order to be able to calculate equilibrium constants from appropriate fluorometric titration data. Fluorescence titration data for normal rabbit  $\gamma$ -globulin (nRyG) and Fab' fragments, Ab-3-Fab', using a large excess of hapten are shown in Fig. 14. The terminal slope of the plot of log (Relative Fluorescence Intensity) against the hapten concentration obtained with Ab-3-Fab' is identical to that found for nRyG. If one uses Beer's Law in the form

$$\log(\%T) = 2 - \varepsilon c$$
 (16)

where  $\varepsilon$  is the molar extinction coefficient of  $\varepsilon$ -DNP-lysine, c the concentration of the hapten and (%T) the percent transmission, it is obvious that the slopes of the two straight lines of Fig. 14 are identical to  $\varepsilon$ . Thus an extinction coefficient of  $1.33 \times 10^4$  $M^{-1}$  cm<sup>-1</sup> at 350 ±5m<sup>µ</sup> was calculated for  $\varepsilon$ -DNP-lysine. This value agrees quite well with the values of  $1.30 \times 10^4$  and  $1.60 \times 10^4$  M<sup>-1</sup> cm<sup>-1</sup> at 345 and 355m<sup>µ</sup>, respectively — for  $\varepsilon$ -DNP-lysine in saline-phosphate buffer (pH 7.4) — which were determined spectrophotometrically. The hapten attenuation in the form 0.0133 log(%T) per µM  $\varepsilon$ -DNP-lysine was used throughout for hapten attenuation corrections. As is evident

Estimation of Q for antibody preparation, Ab-3-Fab' (circles), by titrating with a high concentration of  $\varepsilon$ -DNP-lysine (5 x 10<sup>-4</sup> M). Titration of the control, nR $\gamma$ G (triangles), shows the extent of hapten attenuation.

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from Fig. 14, the control, nRyG, shows no specific quenching by hapten. Extrapolation to zero hapten concentration of the plot for Ab-3-Fab' yielded a Q<sub>max</sub> value of 73.4 per cent (100-antilog 1.425); the corresponding value for the intact antibody, Ab-3, was found to be 65.3 per cent.

After all corrections had been made, data from duplicate titrations of antibody-combining sites with E-DNP-lysine were plotted as shown in Fig. 15. From these specific quenching curves, the concentration of sites at equivalence was obtained as described under "Experimental". In fluorometric titration experiments involving the specifically purified antibody, Ab-3, the concentration of antibodycombining sites titratable with hapten was found to represent 70 per cent of the total concentration of sites present, based on optical density measurements at  $280\,\mathrm{m}\mu$  , the molecular weight of antibody (150,000) and the bivalence of the antibody molecule. These results agree with the findings that the purified antibody preparation was 70 per cent precipitable by DNP-HSA antigen, 30 per cent of the sites apparently contaminated by E-DNP-lysyl groups. Similar results were obtained from fluorometric titrations of the univalent fragments, Ab-3-Fab'. This is to be expected since the procedure for preparation and isolation of the fragments in no instance involved experimental conditions that would bring about the removal of antigen from contaminated sites.

The corresponding binding data, listed in Table VII, were obtained by interpolation on Fig. 15. The Sips plot of log(r/l-r)against log c for the system Ab-3-Fab':  $\varepsilon$  -DNP-lysine is shown in Fig. 16, along with the Sips plot for the system Ab-3:  $\varepsilon$ -DNP-lysine for comparison. All data obtained from fluorometric equilibrium studies are summarized

in Table VIII.

<sup>\*</sup>See Appendix C for the sample treatment of experimental observations to yield corrected titration data.

Specific quenching curve from the titration of Ab-3-Fab' (75  $\mu$ g/ml) with  $\epsilon$ -DNP-1ysine (1 x 10<sup>-4</sup> M).

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Relative Fluorescence Intensity

## Table VII

Binding data for the system Ab-3-Fab' :  $\varepsilon$ -DNP-lysine at 25°C

(Н) <sub>Т</sub> [µМ]	% Quenching, (Q)	r	r/1 - r	с [µM]	log( <u>r'</u> )	log c
0.80	48.2	0.657	1.91	0.09	0.28	-1.04
0.90	51.8	0.706	2.40	0.14	0.38	-0.86
1.00	55.1	0.751	3.01	0.19	0.48	-0.72
1.10	57.7	0.785	3.66	0.25	0.56	-0.60
1.20	59.7	0.813	4.36	0.32	0.64	-0.49
1.30	61.3	0.835	5.06	0.40	0.71	-0.40
1.50	63.7	0.868	6.56	0.56	0.82	-0.25
1.70	65.2	0.888	7.95	0.74	0.90	-0.13
1.90	66.3	0.903	9.33	0.93	0.97	-0.03
2.10	67.0	0.913	10.47	1.11	1.02	0.05
2.30	67.6	0.921	11.64	1.31	1.07	0.11
2.50	68.1	0.928	12.83	1.50	1.11	0.18

Total concentration of binding sites at equivalence, (Ab) $_{\rm T}$ =1.08µM.

Sips plots for fluorescence titration data at  $25^{\circ}$ C for  $\epsilon$ -DNP-lysine with Ab-3 (closed circles) and Ab-3-Fab' (open circles).

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#### Table VIII

Fluorometric Equilibrium Data for the Reaction of Antibody with  $\epsilon$ -DNP-lysine at 25<sup>o</sup>C.

Antibody	Q <sub>max</sub>	Ko [M-1]	α
Ab-3	65.3	2.2 x 10 <sup>7</sup>	0.54
Ab-3-Fab'	73.4	$2.5 \times 10^{7}$	0.70

The binding constants for the antibody preparations, Ab-3 and Ab-3-Fab', are about one order of magnitude lower than those reported for "late" high-affinity antibodies by McGuigan and Eisen (87).  $Q_{\max}$  values are also significantly lower. These differences could be caused by the presence of contaminating antigen which most likely would occupy the sites of highest affinity for antigen and hence would be the most difficult to remove. This would shift the distribution of intrinsic affinity constants toward a lower average value. In addition, the measured  $Q_{\max}$  values would be somewhat lower, since higher affinity preparations have been found to yield correspondingly higher  $Q_{\max}$  values (87).

The results of all equilibrium studies on the three different antibody preparations and their Fab' fragments are summarized in Table IX.

### Table IX

# Equilibrium Data for anti-DNP antibodies and their univalent fragments.

Antibody	Hapten	к <sup>а</sup> [м <sup>-1</sup> ]	α	Method
Ab		1.5 x 10 <sup>5</sup>	0.6	Spectrophotometric
Ab-1 🥆 Fab'	111-2,55-4DNP	$2.3 \times 10^5$	0.5	
Ab		2.7 x 10 <sup>5</sup>		Spectrophotometric
Ab-2 Fab'	1N-2,5S-4DNP	2.9 x 10 <sup>5</sup>		Spec of opriorance of the
Ab		2.2x10 <sup>7</sup>	0.5	Wayon Quonching
Ab-3 Fab'	ε-DNP-lysine	$2.5 \times 10^7$	0.7	FIGHT. Quendining

The most interesting observation, on inspection of Table IX, is that in all cases the Fab' fragments showed a higher affinity for the hapten than the corresponding intact antibody. Slightly higher affinities for hapten by the Fab' fragments were also observed by McGuigan and Eisen (87) and by Nisonoff et al (29). The significance of these observations will be discussed later. The differences in the heterogeneity indices do not appear to be significant enough to warrant special attention.

### Kinetics

### (a) Temperature-Jump

The magnitude of the temperature-jump produced on discharge of the condenser voltage through the solution between the electrodes of the reaction cell was estimated to be  $6^{\circ}C$  (90). The solutions prior to temperature-jumping were therefore equilibrated at  $19^{\circ}C$  in order to obtain kinetic data at  $25^{\circ}C$ . V

# Interaction of 1N-2,5S-4DNP with anti-DNP Ab-1 and Ab-1-Fab'

Rate measurements were performed at 25°C in phosphate buffer (pH 6.0,  $\Gamma/2 = 0.1$ ). Protein and dye solutions of varying concentrations were used to obtain the relaxation time,  $\tau$  , as a function of the sum of the equilibrium concentrations of free antibodycombining sites,  $(\overline{Ab})$ , and free hapten,  $(\overline{H})$ . The experimental conditions used and the relaxation data obtained for the reaction between 1N-2,5S-4DNP and Fab' fragments are shown in Table X. The reciprocal of the relaxation time at a particular concentration of antibody-combining sites and hapten was averaged from 17 observations with an average standard deviation of 11 per cent. The regression of  $1/\tau$  on [(Ab) + (H)] was obtained by "least squares" analysis of the kinetic data for reactions involving both antibody and its univalent fragments. Plots of  $1/\tau$ against [(Ab) +  $(\overline{H})$ ] are shown in Fig. 17. The 95 per cent confidence intervals for the slope  $(k_{12})$  and intercept  $(k_{21})$  of the regression  $1/\tau = k_{21} + k_{12} [(\overline{Ab}) + (\overline{H})]$ (14)

are listed below.

Sustem	$k_{12} [M^{-1} sec^{-1}]$	k_21 [sec <sup>-1</sup> ]	<u>t value</u>
Ab-1: 1N-2.5S-4DNP	$0.95\pm0.13 \times 10^{7}$	76± 36	15
Ab-1-Fab': 1N-2,5S-4DNP	$1.81\pm0.21\times10^{7}$	106± 41	18

It is obvious from the plots of  $1/\tau$  against  $[(\overline{Ab}) + (\overline{H})]$  and the high values of the t distribution, that there is a very significant correlation between  $1/\tau$  and  $[(\overline{Ab}) + (\overline{H})]$  as given by the regression (14) above. The deviations in  $k_{12}$  make overlap of forward rate constant values highly improbable, the forward rate constant for reactions involving the Fab' fragments being significantly higher than that for corresponding reactions involving the whole antibody.

### Table X

Conditions used in relaxation experiments for the system Ab-1-Fab' : 1N-2,5S-4DNP in phosphate buffer (pH 6.0,  $\Gamma/2 = 0.1$ ) at  $25^{\circ}C$ 

(Ab) total x10 <sup>5</sup> [M]	(H) total x10 <sup>5</sup> [M]	(H) bound x10 <sup>5</sup> [M]	(Ab) x10 <sup>5</sup> [M]	(H) x10 <sup>5</sup> [M]	[( <del>Ab</del> ) + (H)] x10 <sup>5</sup> [M]	τ x 10 <sup>3</sup> [sec.]	1/τ [sec <sup>-1</sup> ]
1.33	1.29	0.68	0.65	0.61	1.26	2.92	342 ± 52
1.33	1.61	0.73	0.60	0.88	1.48	2.71	368 ± 48
1.72	1.71	0.82	0.90	0.89	1.79	2.39	419 ± 60
1.67	2.55	0.98	0.69	1.57	2.26	1.90	525 ± 32
1.33	2.51	0.75	0.58	1.76	2.34	1.91	523 ± 59
3.43	3.11	1.94	1.49	1.17	2.66	1.70	588 ± 50

See Appendix A for the statistical treatment of relaxation data obtained for this system.

 $\sum_{i=1}^{n}$ 

The concentration dependence of  $1/\tau$  for the reaction of 1N-2,5S-4DNP with anti-DNP preparations, Ab-1 (open circles) and its univalent fragments, Ab-1-Fab' (closed circles) in phosphate buffer (pH 6.0,  $\Gamma/2 = 0.1$ ) at 25<sup>o</sup>C.



Deviations in the reverse rate constant,  $k_{21}$ , are quite large with a high probability of overlap for the values of  $k_{21}$  obtained with intact antibody and its univalent Fab' fragments.

#### Stopped-flow

Rate measurements were carried out at  $25^{\circ}$ C in the salinephosphate buffer used for fluorometric titrations at concentrations of antibody (Ab-3 or Ab-3-Fab') of about 50 - 150 µg/ml and varying concentrations of the hapten,  $\epsilon$ -DNP-lysine.

Prior to these experiments, the instrument dead time was determined with the aid of the BSA: 1N-2,5S-4DNP system. Fig. 18 shows the increase in transmission of the dye as a function of the time of reaction with BSA. Extrapolation back to the 0 per cent transmission level, obtained by mixing dye and buffer in equal volumes in the stopped-flow apparatus, yielded an average dead time of 10 milliseconds for the system.

The reaction curves for antibody and Fab' fragments with  $\varepsilon$ -DNPlysine were smoothed by hand, and the initial slope at the point of reaction-produced fluorescence dropoff was extrapolated back a distance corresponding to 10 msecs. on the horizontal time scale to yield the 100 per cent fluorescence level at zero time of reaction. A typical reaction curve showing the decrease in fluorescence of the protein as a function of the time of reaction with  $\varepsilon$ -DNP-lysine is shown in Fig. 19. The time (t) was measured as a function of the extent of reaction (x) for each reaction curve, and the data from several reaction curves (Table XI) were analyzed by the "least squares" method. Log  $[(1 - \phi_x)/(1 - x)]$  was plotted as a function of time (Fig. 20)

Reaction curve at 470 m $\mu$  for the BSA:1N-2,5S-4DNP system in phosphate buffer (pH 6.0,  $\Gamma/2 = 0.1$ ) at 25 °C. Sweep rates of 50 and 100 msecs/cm. Upper horizontal line was triggered after the system had reached equilibrium. Lower horizontal line marks the 0 per cent transmission level.

### Figure 19

Reaction curve for the quenching of fluorescence of Ab-3-Fab' (1.55 x  $10^{-6}$  M) by  $\varepsilon$ -DNP-lysine (2.59 x  $10^{-6}$  M) as a function of time. Sweep rate of 20 msecs/cm.

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## Table XI

Kinetic data for the reaction of Ab-3-Fab' with  $\epsilon\text{-DNP-lysine}$  at  $25^{\circ}\text{C}$ 

	·	i	T	r				
Extent of Reaction, $(x)$	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
$\left(\frac{1-\phi x}{1-x}\right)$	1.05	1.11	1.19	1.29	1.44	1.66	2.03	2.76
$\log \left(\frac{1-\phi x}{1-x}\right)$	0.021	0.045	0.075	0.112	0.158	0.220	0.307	0.441
Mean time <sup>*</sup> [msec.], (t)	2.4	5.9	9.1	12.5	17.0	23.1	32.2	47.4
Standard Deviation of observed times	0.3	0.6	0.8	1.2	1.6	2.2	3.5	3.3

\*

Averaged from seven reaction curves.

Initial concentrations of Ab-3-Fab' and  $\varepsilon$ -DNP-lysine were 1.55 x 10<sup>-6</sup> and 2.59 x 10<sup>-6</sup> M, respectively.

 $\phi = 0.56$ 

Kinetics of reaction of  $\epsilon$ -DNP-lysine with the antibody preparation, Ab-3, and its univalent fragments, Ab-3-Fab' at 25°C, plotted according to equation (15). Initial concentration of Ab-3, 2.0 x 10<sup>-6</sup> M; of hapten, 2.3 x 10<sup>-6</sup> M. Initial concentration of Ab-3-Fab', 1.6 x 10<sup>-6</sup> M; of hapten, 2.6 x 10<sup>-6</sup> M.


according to the equation:

$$\log \left(\frac{1-\phi_x}{1-x}\right) = \frac{k_{12}\sqrt{-Q}}{2.303} t$$
 (15)

The model for the regression of  $\log [(1 - \phi_x)/(1 - x)]$  on t:

$$\log \left(\frac{1-\phi x}{1-x}\right) = c + \frac{k_{12}\sqrt{-Q}}{2.303} t \qquad (17)$$

was chosen to test the estimation of 10 msecs. for the system dead time; according to equation (15), plots of log  $[(1 - \phi_x)/(1 - x)]$ against t should go through the origin. On the average values of C varied between ±1 millisecond about the origin; this is insignificant for reaction time observations up to 50 - 100 milliseconds.

Plots of log  $[(1 - \phi x)/(1 - x)]$  against time were, on the whole, linear yielding single values for the forward rate constant, k<sub>12</sub>. Under the conditions of initial concentrations of hapten and antibodycombining sites used, it was calculated that 80 per cent of the sites were occupied at equilibrium. Yet no kinetic heterogeneity was observed up to about 80 per cent completion of the reaction. Similar results were found by Day et al (95). These authors observed deviations from linearity in their kinetic plots only when concentrations of hapten in a two-fold excess over the concentration of antibody sites were used, and this downward curvature away from the initial slope began approximately when the reaction was 80 per cent complete. The precise value of the average intrinsic dissociation constant,  $K_{O}^{a}$ , is not a critical factor for the determination of the limiting rate constants. For instance, substitution into the rate equations of  $K^{d}_{\alpha}$ values differing by a factor of 3 yielded forward rate constants which differed only by 15 per cent. It would seem, therefore, that the

dissociation reaction is of no great consequence until the reaction is near completion, and then only when the sites of weakest affinity for hapten are involved in the reaction, as would be the case under conditions of hapten excess.

The kinetic data from temperature-jump and stopped-flow studies are summarized in Table XII. The values of  $k_{12}$  are in the range (  $\sim 10^7 - 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ ) usually found for hapten-antibody reactions (Table III, Chapter II). A comparison of the data for the rates of reaction of the two anti-DNP antibody preparations with the different dinitrophenyl haptens is of particular significance. Although the equilibrium constants for the binding of 1N-2,5S-4DNP were about two orders of magnitude lower than those obtained for the binding of  $\epsilon$ -DNP-lysine, the forward rate constants were about the same. Thus, the binding constant of a hapten-antibody reaction appears to be governed mainly by the 'lifetime' of the complex and not by the rate at which the two reactants can combine.

It is evident from Table XII that the rate of association of hapten with Fab' fragment is about twice as fast as the rate of the corresponding association with the parent antibody molecule. Differences in the rates of dissociation, however, are not as pronounced In fact, from the deviations of  $k_{21}$  values obtained from temperature-jump studies, it is quite possible that the reverse constants may actually be the same for the dissociation of haptenantibody and hapten-Fab' complexes. Unfortunately, the reverse rate constants could not be obtained independently by the stopped-flow method.

The significance of these equilibrium and kinetic results are discussed in terms of experimental error in Appendix D.

## Table XII

Kinetic data for the reaction of antibody and Fab' fragments with hapten at 25°C

Antibody	Hapten	k <sub>12</sub> [M <sup>-1</sup> sec <sup>-1</sup> ]	<sup>k</sup> 21 [sec <sup>-1</sup> ]	$k_{12}^{k}/k_{21} = K_0^a [M^{-1}]$	Method	
Ab.		$0.95 \pm 0.18 \times 10^7$	76 ± 36	1.3 x 10 <sup>5</sup>	-	
Ab-1	1N-2,5S-4DNP	7		5	T-Jump	
Fab'		$1.81 \pm 0.20 \times 10'$	$106 \pm 41$ $1.7 \times 10^{-1}$	$1.7 \times 10^{-1}$		
Ab		$1.10 \pm 0.20 \times 10^7$	0.51 <sup>a</sup>	n.d. <sup>b</sup>		
Ab-3 Fab'	ε-DNP-lysine	$2.00 \pm 0.20 \times 10^7$	0.80 <sup>a</sup>	n.d. <sup>b</sup>	Stopped-Flow	

<sup>a</sup> Determined from the relationship  $k_{21} = k_{12}/K_0^a$ 

<sup>b</sup> Could not be determined independently.

Values of  $k_{12}$  and  $k_{21}$  listed are their 95 per cent confidence intervals.

#### CHAPTER IV

## KINETICS OF THE REACTIONS OF ANTIBODY AND ITS POLYPEPTIDE CHAINS WITH HAPTEN

#### INTRODUCTION

Immunoglobulins of the IgG class are composed of two types of chains, two heavy and two light chains. It is not clear yet whether the antibody-combining site is present in only one of the chains or whether both chains are required for the formation of the site. This subject has been discussed at length in the first chapter of this thesis.

Various experiments have demonstrated that the isolated heavy chains still contain part of the antibody activity, as well as preserve the specificity of binding present in the original antibody (36,60). This antibody activity was shown to be confined to the Fd region of the heavy chain (61). However, the evidence presented has been mainly in terms of the stoichiometry of the reaction and the affinity of the heavy chain relative to that of the intact antibody for homologous hapten.

This study was undertaken in an attempt to extend the investigation of the antibody activity residing in the isolated heavy chain to the kinetics of its reaction with hapten. One of the aims of this study was to determine whether or not the reaction of heavy chains with hapten could still be described by the simple one-step mechanism

Ab + H 
$$\xrightarrow{k_{12}}_{k_{21}}$$
 AbH (1)

which has been shown, in the previous chapter, to hold true for the reactions of antibody and its Fab' fragments with hapten. Also, from a comparison of the forward and reverse rate constants for reactions involving the intact antibodies with the rate constants for the reactions involving their component heavy chains, it might be possible to draw some conclusions as to the rigidity and structure of the antibody-combining site still contained in the isolated heavy chains. Moreover, it was anticipated that such a comparison of these rate constants might yield some indirect evidence with regard to the role played by the light chains in the formation of the antibody-combining site.

The 2,4-dinitrophenyl system was chosen since (i) binding data for the isolated heavy chains had already been reported (60,61), and (ii) the system could be studied by the highly sensitive technique of fluorescence quenching. A very sensitive method of measuring quantitative binding of hapten is necessary because of the rather low binding constants reported for the reaction of hapten with specific heavy chains.

#### EXPERIMENTAL

#### Preparation of Immunoglobulins

The antibody preparation used in this study was that referred to as Ab-3 in the previous chapter. Normal rabbit  $\gamma$ -globulin (nR $\gamma$ G), used in control experiments, was obtained from the anti-DNP antiserum after the specific antibody, Ab-3, had been completely removed with the immunosorbent, DNP-HSA-BAC. The nR $\gamma$ G preparation was extracted by sodium

sulfate precipitation and purified by chromatography on DEAE-cellulose (Whatman DE23, microgranular, l = equiv/g) in phosphate buffer (pH 7.6,  $\Gamma/2 = 0.02$ ) at room temperature. The purified nRyG showed no antibody activity when tested by quantitative precipitin and fluorescence quenching techniques.

## Preparation of Mildly Reduced, Alkylated and Polyalanylated IgG

The procedure followed for the preparation of mildly reduced, alkylated and polyalanylated nRyG and Ab-3 was essentially that described by Jaton et al (61) with slight modifications. Samples (150 mg) of IgG were dialyzed against 0.1 M Tris-HCl buffer (pH 8.0) and reduced with 0.075 M freshly distilled 2-mercaptoethanol (Eastman Chemicals, New York) under nitrogen at room temperature. After alkylation with recrystallized iodoacetamide (Koch-Light Laboratories Ltd., Bucks, England) at 10 per cent molar excess over the 2-mercaptoethanol, the solution was dialyzed against 0.05 M phosphate buffer (pH 7.0) for 24 hours. The solution was then used for polyalanylation according to the method of Fuchs and Sela (135), using a 1:1 weight ratio of N-carboxy-DL-alanine anhydride (Miles-Yeda Ltd., Rehovoth, Israel) to IgG. The mildly reduced, alkylated and polyalanylated  $\gamma$ -globulin (RAP-Ab-3, RAP-nR $\gamma$ G) was then finally dialyzed against 4 changes of 1 M prop ionic acid (500 ml each) for 48 hours.

### Fractionation of RAP-7-globulin

After dialysis against 1M prop ionic acid, the reduced, alkylated and polyalanylated IgG solution (15 ml, 10 mg/ml) was applied to a Sephadex G-100 M column (2.5x170 cm) equilibrated with 1 M prop ionic acid at 5°C (35). The nonaggregated heavy chain fraction only was pooled, adjusted to neutral pH, dialyzed exhaustively against distilled water and finally lyophilized. The light chain fraction was similarly treated.

#### Physico-Chemical Characterization of Polypeptide Chains

Amino acid analyses of the heavy chains, RAP-nRyG-H, obtained from the reduced, alkylated and polyalanylated  $\gamma$ -globulin preparation were performed on a Beckman automatic amino acid analyzer, model 120B, after hydrolysis under reduced pressure in constant boiling hydrochloric acid (6N) for 22 hours at 110°C (136). In order to determine the number of amino groups which acted as initiators in the polymerization reaction to yield the polyalanylated derivative, RAP-nRyG-H, the derivative was treated with sodium nitrite in glacial acetic acid (137). The number of alanylated lysine residues (which were not deaminated) was determined by quantitative amino acid analysis. The enrichment in alanine residues by polyalanylation was computed assuming 33.7 residue moles of aspartic acid and 6.9 residue moles of histidine per mole of heavy ( $\gamma$ ) chain (37).

The molecular weights of the polyalanylated heavy and light chains were estimated from their elution volumes on gel filtration through a Bio-Gel P-200 column (1.5 x 90 cm) equilibrated in 5 M guanidine-HCl (Mann Research Laboratories, New York) at room temperature according to Andrews (138). The column was calibrated with the standards: reduced and alkylated BSA (mol. wt. 68,000), rabbit  $\gamma$ chains (mol. wt. 53,000) and rabbit light chains (mol. wt. 22,500). The calibration curve was prepared by plotting the square root of the distribution coefficients, K<sub>D</sub>, of the standards against the square root of their molecular weights (139); the distribution coefficient is

related to the elution volume,  $V_e$ , by:

$$V_e = V_o + K_D V_i$$

where  $V_{o}$ , the void volume, is the elution volume of a solute completely excluded from the internal cavities of the gel, and  $V_{i}$  is the volume of solvent imbibed by the gel. Gel filtration in 5 M guanidine-HCl was used simultaneously to test the purity of the polyalanylated heavy chains for contamination by light chains.

Sedimentation coefficients of polypeptide chain preparations were determined using a Beckman Model E ultracentrifuge, equipped with schlieren optics. Samples were sedimented in (a) phosphate buffered saline (pH 7.4) and (b) 0.04 M sodium decyl sulfate, 0.20 M NaCl, 0.01 M phosphate buffer (pH 7.7) at 50,740 rpm. The factors used to convert the observed sedimentation values to  $s_{20,w}$  were 1.045 and 1.105 (36) for solvents (a) and (b), respectively.

Concentrations of the antibody preparations: Ab-3,RAP-Ab-3 and RAP-Ab-3-H, in solution at pH 7.4, were determined from optical density measurements at  $280_{m\mu}$  in a Zeiss PMQII spectrophotometer, using an extinction coefficient ( $E_{lcm}^{1\%}$ ) of 15 for all three preparations, since the values of their respective extinction coefficients, reported by Jaton et al (61), did not differ by more than 3 per cent.

#### Equilibrium Studies

The binding of  $\varepsilon$ -DNP-lysine by mildly reduced, alkylated and polyalanylated antibody (RAP-Ab-3), heavy chains (RAP-Ab-3-H), and light chains (RAP-Ab-3-L) was determined experimentally by the method of fluorescence quenching in an Aminco-Bowman spectrophotofluorometer as described in the preceding chapter. In  $Q_{max}$  determinations, the antibody derivatives were titrated with a high concentration  $(1 \times 10^{-3} \text{ M})$ of  $\varepsilon$ -DNP-lysine to a final concentration of hapten of about a 60-80 fold molar excess over antibody site concentration to approach saturation of the antibody-combining sites. As controls, normal rabbit  $\gamma$ -globulin (nRyG) and its reduced, alkylated and polyalanylated heavy chains (RAP-nRyG-H) and light chains (RAP-nRyG-L) were used. The concentrations of protein used in the fluorometric titrations were of the order of 50 - 150 µg/ml in 0.15 M NaCl, 0.01 M phosphate buffer (pH 7.4). All titrations were performed in duplicate, using various concentrations of protein. The protein samples were excited at 290mµ and the emitted fluorescence intensity was measured at 350mµ.

The measured fluorescence intensity was corrected for dilution, solvent blank and hapten attenuation. The binding data were then analyzed to yield binding site concentrations at equivalence, valences, average intrinsic association constants and heterogeneity indices as described in the previous chapter under "Experimental". Kinetic Studies

Rate measurements of the quenching of antibody fluorescence by  $\epsilon$ -DNP-lysine were performed at 25°C, using the stopped-flow spectrophotometer. The investigations were carried out in 0.15 M NaCl, 0.01 M phosphate buffer (pH 7.4) at protein concentrations of 150 µg/ml and varying concentrations of hapten.

The kinetic data were analyzed by the "least squares" method according to the rate equation

$$\log\left(\frac{1-\phi_x}{1-x}\right) = \frac{k_{12}\sqrt{-Q}}{2\cdot 303}$$
 t (15)

#### Relative Quantum Yield

The quantum yield of the antibody preparations: Ab-3, RAP-Ab-3 and RAP-Ab-3-H in phosphate-buffered saline (pH 7.4) relative to the standard, L-tryptophan (Sigma Chemical Co.), in double-distilled water was determined according to the method of Teale and Weber (140). Solutions of known absorbance at 280mµ were excited at 290mµ, and their emission spectra in the wavelength range of 300-460mµ were recorded. The relative quantum yields ( $Q_r$ ) were calculated according to equation (18):

# $Q_r = \frac{\text{Area under the emission spectrum of the sample}}{\text{Area under the emission spectrum of tryptophan}}$ , per unit

of absorbance at 280mµ. Only tryptophan fluorescence was considered because of the small tyrosine contributions; also the emission spectra were not corrected for variation of instrumental sensitivity with wavelength, since the sensitivity was constant within ±10 per cent over the wavelength range of the fluorescence spectra obtained (141).

#### RESULTS AND DISCUSSION

#### Polyalanylated Polypeptide Chains

The elution pattern for the fractionation of reduced, alkylated and polyalanylated anti-DNP antibodies on a Sephadex G-100 column in 1 M prop ionic acid is shown in Fig. 21. The recovery of light chains was 26 per cent of the total absorbancy at  $280m\mu$ , which is in good agreement with the theoretical yield of 30 per cent. The isolated non-aggregated heavy chain component was rerun on Sephadex G-100 in 1M prop ionic acid and showed less then 2 per cent contamination by light chains.

Amino acid analysis of the polyalanylated heavy chains, RAP-nRyG-H, before and after deamination showed that 14 of the 24 lysine residues of the heavy ( $\gamma$ ) chains reacted with N-carboxy-DLalanine anhydride, forming sidechains of 6-7 alanine residues each [Table XIII]. From the alanine enrichment of the heavy chains, the molecular weight was estimated to be 60,000 ±800, based on a molecular weight of 53,000 for the untreated heavy ( $\gamma$ ) chains. Jaton et al (61) estimated the molecular weight of their polyalanylated heavy chains to be about 56,000, presumably on the basis of 50,000 for the molecular weight of  $\gamma$  chains, since the extent of alanine enrichment was approximately the same as reported in this study.

The elution pattern of mildly reduced, alkylated and polyalanylated antibodies (150 mg) from a Sephadex G-100 column (2.5 x 170 cm) equilibrated with 1M propi onic acid at 4<sup>o</sup>C.



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#### Table XIII

Sample	Alanine	Aspartic Acid	Total a Added b Alanine a Alanine		Average Alanine c	
	µmoles/ sample	µmoles/ sample	residues/ molecule	residues/ molecule	residues/ sidechain	
RAP-nRyG-H	1.270	0.349	127	104	7	
Deaminated RAP-nRyG-H	0.910	0.315	105	82	6	

#### Alanine enrichment in polyalanylated heavy chains.

<sup>a</sup> Assuming 33.7 aspartic acid residues per heavy chain (37)

<sup>b</sup> Assuming 22.9 alanine residues per heavy chain (37)

<sup>C</sup> The number of sidechains per heavy chain was assumed to be identical with the number of alanylated lysine residues.

A sample (12 mg/ml) of polyalanylated heavy chains (RAP-Ab-3-H) obtained from anti-DNP antibodies was chromatographed on Bio-Gel P-200 in 5 M guanidine-HCl [Fig. 22], after dialysing against 5 M guanidine-HCl for 3 days. The extent of contamination by light chains was estimated at about 5-6 per cent of the total absorbancy at  $280m\mu$  This value is in good agreement with the 4-5% light chain contamination reported by Utsumi and Karush (36) and somewhat higher than the lower limit of 1-2% observed by Jaton et al (61), on the basis of immunological methods. The molecular weights of the polyalanylated heavy and light chains were estimated to be 61,000 and 28,000, respectively [Fig. 23]. The estimated value for the molecular weight of the polyalanylated heavy chain by this method is in excellent agreement with the value determined from amino acid analysis.

The elution pattern of polyalanylated antibody heavy chains, RAP-Ab-3-H (12 mg), from a Bio-Gel P-200 column (1.5 x 90 cm) equilibrated with 5M guanidine-HCl (pH 8.0) at room temperature.

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Calibration curve of  $(K_D)^{\frac{1}{2}}$  against  $(M.W.)^{\frac{1}{2}}$  for the estimation of the molecular weights of polyalanylated heavy (RAP-Ab-3-H) and light (RAP-Ab-3-L) chains. The distribution coefficients, K , were determined D from the respective elution volumes on a Bio-Gel P-200 column equilibrated with 5M guanidine-HC1.



The molecular weights were taken as 176,000 and 60,000 for the reduced, alkylated and polyalanylated antibody (RAP-Ab-3) and its component heavy chain (RAP-Ab-3-H), respectively.

Ultracentrifugal analysis of the isolated alanylated heavy chains (10 mg/ml) in detergent buffer showed a single peak with a sedimentation coefficient  $(s_{20,w})$  of 3.6S [Fig. 24a]. However, sedimentation of the polyalanylated heavy chains (3 mg/ml) in phosphate buffered saline (pH 7.4) revealed a single component with an  $s_{20,w}$  value of 5.4S [Fig. 24b]. These results indicate that, in the absence of dissociating agents, the polalanylated chains dimerize in solution at the concentrations used for sedimentation analysis. Polyalanylation, therefore, served its purpose in preventing the formation of multiple aggregates of heavy chains, thereby increasing their solubility in neutral aqueous buffer considerably (135). At the same time the affinity of antibody for hapten was not significantly decreased, as will be shown in the following section.

## Effects of Mild Reduction, Alkylation and Polyalanylation on Antibody Activity.

Before the residual activity in the isolated polypeptide chains could be compared with that of the parent antibody, the effects of reduction and polyalanylation of the whole antibody (Ab-3) on its specific activity had to be predetermined.

The binding of  $\varepsilon$ -DNP-lysine by the antibody preparation, RAP-Ab-3, was investigated by the method of fluorescence quenching. The specific quenching of antibody fluorescence as a function of hapten concentration is shown in Fig. 25. A comparison of the antibody activity of the mildly reduced, alkylated and polyalanylated antibody with that of the untreated antibody is given in Table XIV.



## Figure 24a

Sedimentation pattern of RAP-Ab-3-H (10 mg/ml) in 0.04M sodium decylsulfate, 0.20M NaCl, 0.01 M phosphate buffer (pH 7.7) at 50,740 rpm ( $20^{\circ}$ C) in a synthetic boundary cell. Photographs were taken at 8 minute intervals.



## Figure 24b

Sedimentation pattern of RAP-Ab-3-H (3 mg/ml) in 0.15M NaCl, 0.01M phosphate buffer (pH 7.4) at 50,740 rpm ( $20^{\circ}$ C) in a synthetic boundary cell. Photographs were taken at 8 minute intervals.

100



## Figure 24a

 $\sum_{i=1}^{n}$ 

Sedimentation pattern of RAP-Ab-3-H (10 mg/ml) in 0.04M sodium decylsulfate, 0.20M NaCl, 0.01 M phosphate buffer (pH 7.7) at 50,740 rpm ( $20^{\circ}$ C) in a synthetic boundary cell. Photographs were



## Figure 24b

Sedimentation pattern of RAP-Ab-3-H (3 mg/ml) in 0.15M NaCl, 0.01M phosphate buffer (pH 7.4) at 50,740 rpm ( $20^{\circ}$ C) in a synthetic boundary cell. Photographs were taken at 8 minute intervals.

Specific quenching curves for the reaction of  $\epsilon$ -DNP-lysine with the antibody preparation, RAP-Ab-3, at concentrations of 0.89  $\mu$ M (closed circles) and 0.36  $\mu$ M (open circles) in 0.15 M NaCl, 0.01 M phosphate buffer (pH 7.4) at 25°C.



#### Table XIV

Antibody	Q <sub>max</sub>	к <sub>о</sub> а [м <sup>-1</sup> ]	α	n
Ad-3	65.3	2.16 x 10 <sup>7</sup>	0.54	2.1
RAP-Ad-3	42.5	1.07 x 10 <sup>7</sup>	0.44	1.6

Binding of  $\varepsilon$ -DNP-lysine by antibody at 25°C.

Thus, reduction and polalanylation has caused a 50 per cent reduction in the affinity constant. This is in exact agreement with observations of Karush and Sela (142) and much less than the 80 per cent reduction observed by Jaton et al (61). Also, the loss of binding sites as a consequence of the preparative treatment is slightly more than reported by Jaton et al. The reductions in binding constant and  $Q_{max}$  values are probably due to loss of rigidity of the binding site. The increased heterogeneity might have arisen if all of the sites were not equally affected.

#### Antibody Activity in the Polypeptide Chains

Since earlier reports have presented conflicting evidence for hapten binding by the individual heavy and light chains (58-69), the quenching of their fluorescence by hapten was carefully screened by duplicate and, in some cases, triplicate experiments using at least two different concentrations of protein for the titration. The corrected quenching data for the specific chains were carefully compared with the data for the corresponding chain preparations from inert purified nRyG. The results of titration of several preparations with  $\varepsilon$ -DNP-lysine are illustrated graphically in Fig. 26. These preparations include normal  $\gamma$ -globulin (nR $\gamma$ G), its polyalanylated (RAP-nR $\gamma$ G-L) and non-polyalanylated (RA-nR $\gamma$ G-L) light chains and the polyalanylated light chains (RAP-Ab-3-L) from specific antibody (Ab-3). None of these preparations showed any antibody activity. Plots of the logarithm of the relative fluorescence intensity against hapten concentration are linear and identical for all four preparations. The reduction in fluorescence intensity is due solely to hapten attenuation. Thus, the technique of fluorescence quenching, despite its extreme sensitivity, could not detect any antibody activity in the light chains of anti-DNP antibody. Similar observations were made by Jaton et al (61) and Haber and Richards (60) using equilibrium dialysis and fluorescence guenching techniques.

The polyalanylated specific heavy chains (RAP-Ab-3-H) on the other hand did show some residual activity, as is evident from Fig. 27. Correction for hapten attenuation by extrapolation of the limiting slope at excess hapten concentrations to zero hapten concentrations yielded a  $Q_{max}$  value of 21±1 per cent. This was an average of the values obtained from two series of duplicate titrations of heavy chains with  $\varepsilon$ -DNP-lysine  $(1 \times 10^{-3} \text{ M})$  using protein concentrations of 34 and 53 µg/ml. It should be pointed out that the method of plotting the logarithm of the relative fluorescence intensity against the concentration of hapten, adopted in this study, yields more reliable values of  $Q_{max}$  than the conventional method (94). In the conventional method, fluorescence values, corrected for dilution and solvent blank, are plotted as a function of hapten concentration,

Fluorometric titration of the preparations: nRyG (closed circles), RAP-nRyG-L (open circles), reduced and alkylated nRyG light chains (closed triangles) and specific light chains, RAP-Ab-3-L (open triangles), with  $\varepsilon$ -DNP-lysine (lx10<sup>-3</sup> M) at 25<sup>o</sup>C.



Estimation of Q from duplicate titrations of polyalanylated specific heavy chains, RAP-Ab-3-H (34 µg/ml, circles) with  $\varepsilon$ -DNP-1ysine (1.06 x 10<sup>-3</sup> M). The control, RAP-nR $\gamma$ G-H (triangles) showed no specific activity. Titrations were performed in 0.15 M NaCl, 0.01 M phosphate buffer (pH 7.4) at 25°C.



and the pseudolinear terminal slope at excess hapten concentrations is extrapolated back to zero hapten concentration to determine Q<sub>max</sub>. Thus, the present method also yields more precise data as to the total number of combining sites present in a given antibody preparation. This is particularly true if antibodies or, as in the present case, heavy chains of low affinity are being titrated.

## Stoichiometry and Equilibrium Parameters.

More concentrated solutions (150  $\mu$ g/ml) of specific heavy chains, RAP-Ab-3-H, were titrated with small aliquots of a diluted hapten solution (2 x 10<sup>-4</sup> M) in duplicate. After corrections for dilution, solvent blank and hapten attenuation were made, the relative fluorescence intensity was plotted as a function of hapten concentration. Several curves were drawn by successive approximations through the specific quenching data, until a quenching curve was obtained which fitted the experimental points and, at the same time, yielded a linear Sips plot. The number of combining sites, [Ab]<sub>T</sub>, in the heavy chain solution was estimated from this "best fitting" quenching curve [Fig. 28].

The number (n) of combining sites per heavy chain obtained from several titrations varied between 0.40 and 0.50 sites. This value is only an approximation because of the uncertainty in positioning of the limiting slope at low hapten concentration and the 5 per cent deviation in  $Q_{max}$ . The average value for n(0.45 sites per heavy chain) is somewhat higher than the value of 0.26 sites, reported by Jaton et al (61) with the aid of the equilibrium dialysis technique. However, it is possible that sites of very low affinity may escape detection by the

Specific quenching curve for the reaction of  $\varepsilon$ -DNP-lysine (2.2x10<sup>-4</sup> M) with specific heavy chains, RAP-Ab-3-H (157 µg/ml) at 25°C, pH 7.4.



method of equilibrium dialysis. The fact that only 0.45 sites were found per heavy chain, as compared to 0.8 sites per Fab moiety of the reduced, alkylated and polyalanylated but otherwise intact antibody molecule, would indicate that some sites, most likely those of low affinity, were destroyed upon separation of the component polypeptide chains. Alternatively, the affinity for hapten of some of the heavy chains could have been reduced so drastically that it escaped detection.

Sips plots for the reactions of RAP-Ab-3 and RAP-Ab-3-H with  $\epsilon$ -DNP-lysine are shown together in Fig. 29 for purposes of comparison. It is evident that a further slight increase in the heterogeneity of affinity of the heavy chain sites for hapten has occurred. The effects of the 5 per cent deviation in  $Q_{max}$  and the corresponding deviation in the conentration of antibody sites,  $[Ab]_{T}$ , at equivalence on the calculated value of  $K_{o}^{a}$  were determined by substituting the corresponding values of the constants,  $Q_{max}$  and  $[Ab]_{T}$ , in the calculations of log (r/l - r) and log c [Table XV]. It was found that the 5 per cent deviation in  $Q_{max}$  produced a 25 per cent deviation in the binding constant,  $K_{o}^{a}$ . The equilibrium parameters calculated for the interaction of RAP-Ab-3-H with  $\epsilon$ -DNP-lysine are listed below:

$$K_o^a = 3.8 \pm 1.0 \times 10^5 \text{ M}^{-1}$$
  
 $\alpha = 0.39$ 

Thus, the affinity constant for the reaction between heavy chains and hapten decreased 60-fold as compared with the reaction involving the intact parent antibody. Jaton et al (61) had observed a 100-fold decrease in the binding constant. The greater reduction

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Quenching data for the binding of  $\epsilon\text{-DNP-lysine}$  by RAP-Ab-3-H at  $25^{\circ}\text{C}$  .

(H) <sub>T[µm]</sub>	% Quenching, (Q).	r	(r/1-r)	log (r/1-r)	c [µm]	log c
0.50	5.5	0.262	0.36	-0.45	0.15	-0.81
1.00	7.4	0.352	0.54	-0.27	0.54	-0.27
1.50	8.5	0.405	0.68	-0.17	0.97	-0.02
2.00	9.3	0.443	0.80	-0.10	1.42	0.15
3.00	10.3	0.490	0.96	-0.02	2.35	0.37
4.00	11.0	0.521	1.09	0.04	3.31	0.52
5.00	11.5	0.548	1.21	0.08	4.28	0.63
7.00	12.2	0.581	1.39	0.14	6.23	0.80
9.00	12.7	0.605	1.53	0.19	8.20	0.91
11.00	13.1	0.624	1.66	0.22	10.18	1.01
15.00	13.7	0.652	1.87	0.27	14.14	1.15

 $Q_{max} = 21\%$ 

(AB)<sub>T</sub>= 1.32 µM.

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Sips plots for fluorescence titration data at  $25^{\circ}C$  for  $\epsilon$ -DNP-lysine with RAP-Ab-3 (circles) and RAP-Ab-3-H (triangles).


in affinity observed by these authors may have been due to their more drastic conditions for reduction of antibody. They used 0.1 M 2-mercaptothanol as compared to 0.075 M employed in this study.

## Kinetic Studies

# Reaction of RAP-Ab-3 with &-DNP-lysine

Rate measurements of the reaction of reduced, alkylated and polyalanylated antibody (RAP-Ab-3) with hapten were carried out at 25°C in phosphate-buffered saline (pH 7.4) with the aid of the stopped-flow spectrophotometer.

The initial concentration of combining sites was calculated from the protein concentration, based on optical density measurements at 280 mµ, and the number of sites (n) per mole of total protein, obtained from fluorometric titrations. The extent of quenching of antibody fluorescence was measured as a function of time and analyzed according to the rate equation:

$$\log \left(\frac{1-\phi x}{1-x}\right) = \frac{k_{12}}{2.303} t$$
 (15)

as described in the preceding chapter. Log  $[(1-\phi_x)/(1-x)]$  was plotted against time, and the best fitting straight line was obtained by the method of least squares. [Fig. 30].

A very interesting observation was that the rate constant of association  $(k_{12})$  for this system was calculated to be  $1.93 \pm 0.20 \times 10^7$   $M^{-1} \, \mathrm{sec}^{-1}$ , which is almost identical to that found for the corresponding reaction involving Fab' rather than untreated antibody. The rate constant of dissociation  $(k_{21})$  was of the order of 1.80 sec<sup>-1</sup>. Thus,

# Figure 30

Kinetics of reaction of  $\varepsilon$ -DNP-lysine (2.80 x 10<sup>-6</sup> M) with RAP-Ab-3 (1.27 x 10<sup>-6</sup> M) at 25°C plotted according to equation (15).



the dissociation reaction was two to three times faster than that observed for the intact antibody-hapten complex.

# Reaction of heavy chain, RAP-Ab-3-H, with &-DNP-lysine

The rate of the forward reaction for this system was extremely slow even at the highest concentrations of reactants. The halftimes of the reaction between the isolated heavy chains and hapten ranged from 1-9 seconds, depending on the concentrations of reactants used. As a result, considerable difficulty was experienced in obtaining smooth reaction curves, due to the small signals corresponding to the change in fluorescence intensity, and due to fluctuations in lamp intensity. The inherent instability of the lamp posed the more serious problem since it could not be rectified; fluctuations in lamp intensity rarely caused complications when reactions having half-times of about 20 milliseconds were followed.

Typical reaction curves for the specific quenching of antibody fluorescence by hapten as a function of time are shown in Fig. 31. Several reaction curves were photographed, using three different combinations of initial concentrations of reactants: the concentrations of specific heavy chains in terms of antibody-combining sites were about  $1.3 \times 10^{-6}$  M, with  $\varepsilon$ -DNP-lysine concentrations in the range of  $4.6 \times 10^{-6}$  to  $16.5 \times 10^{-6}$  M. The reaction curves were smoothed by hand, and the extent of reaction as a function of time was analyzed by the 'least squares' method according to equation (15).

A typical plot of log  $[(1-\phi x)/(1 - x)]$  against t for the system RAP-Ab-3-H:  $\varepsilon$ -DNP-lysine is shown in Fig. 32. The linearity of the plot indicates that the reaction can be represented by an

# Figure 31

Typical reaction curves for the quenching of antibody fluorescence by  $\varepsilon$ -DNP-lysine as a function of time at 25°C: (a) [RAP-Ab-3] =  $1.27 \times 10^{-6}$  M, [ $\varepsilon$ -DNP-lysine] = 2.79  $\times 10^{-6}$  M; (b) [RAP-Ab-3-H] =  $1.17 \times 10^{-6}$  M, [ $\varepsilon$ -DNP-lysine] =  $4.6 \times 10^{-6}$  M.



(b) RAP-Ab-3-H +  $\epsilon$ -DNP-1ysine, 500 msec/cm.



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(a) RAP-Ab-3 +  $\varepsilon$ -DNP-lysine, 50 msec/cm.



(b) RAP-Ab-3-H +  $\epsilon$ -DNP-lysine, 500 msec/cm.

# Figure 32

Kinetics of reaction of  $\varepsilon$ -DNP-lysine (8.33 x 10<sup>-6</sup> M) with RAP-Ab-3-H (1.3 x 10<sup>-6</sup> M) at 25°C plotted as a second order forward, first order reverse reaction [equation (15)].



expression of the form:

$$Ab + H \xrightarrow{k_{12}} AbH$$

Moreover, it would appear that there are no deviations from linearity due to the heterogeneity of the heavy chain preparation. However, it is quite possible that such deviations were obscured by the large errors involved in these rate determinations.

#### Table XVI

Kinetic data for the reaction of RAP-Ab-3-H with &-DNP-lysine at 25°C.

Initial Concentration x10 <sup>6</sup> [M]		k <sub>12</sub> [M <sup>-1</sup> sec <sup>-1</sup> ]	$k_{21} \left( = \frac{k_{12}}{m_{12}} \right) [sec^{-1}]$
RAP-Ab-3-H	ε-DNP-lysine		K <sub>o</sub> -
1.34	16.5	$3.4 \times 10^4$	0.09
1.31	8.3	$2.3 \times 10^4$	0.06
1.17	4.6	$2.0 \times 10^4$	0.05

The limiting rate constants in Table XVI are probably accurate to  $\pm 50$  per cent. The uncertainties in the value of n, used to calculate the initial binding site concentrations, and in the association constant  $(K_0^{a})$  used in the calculation of the parameters  $\phi$  and  $\sqrt{-Q}$  in the rate equation, were not even considered because of the much larger error in the kinetic measurements themselves. As a consequence, all one can say is that the forward rate constant for the heavy chain - hapten interactions are of the order of  $10^4 \text{ M}^{-1} \text{ sec}^{-1}$ , three orders of magnitude lower than the rate constant for the reactions involving the intact binding site in the preparations: Ab-3, Ab-3-Fab' and RAP-Ab-3 (see summary Table XVII). The reverse rate constant was on the average, about one order of magnitude lower than that for the dissociation of the  $\varepsilon$ -DNP-lysine: Ab-3 complex, so that the lifetime of the bound hapten in the binding site of the heavy chain is considerably longer. The drastic reduction in the association rate constant indicates a significant loss of rigidity of the heavy chain binding site.

### Table XVII

Antibody $k_{12} [M^{-1} \text{ sec}^{-1}]$  $k_{21} [\text{sec}^{-1}]$ Ab-3 $1.10 \pm 0.20 \times 10^7$ 0.51Ab-3-Fab' $2.00 \pm 0.20 \times 10^7$ 0.80RAP-Ab-3 $1.93 \pm 0.13 \times 10^7$ 1.80RAP-Ab-3-H $3.0 \pm 1.5 \times 10^4$ 0.07

Kinetic data for  $\varepsilon$ -DNP-lysine : antibody reactions at 25°C.

## Relative Quantum Yields

The quantum yields,  $Q_r$ , of the antibody preparations: Ab-3, RAP-Ab-3 and RAP-Ab-3-H in phosphate buffered saline (p. 7.4), relative to the tryptophan standard in double-distilled water, are listed in Table XVIII along with their wavelengths of maximum fluorescence,  $\lambda_{max}$ .

## Table XVIII

Antibody	Ab-3	RAP-Ab-3	RAP-Ab-3-H
Q <sub>r</sub>	0.23	0.33	0.43
λ ,mµ max	341	342	345

Quantum yields and  $\lambda_{max}$  of fluorescence of anti-DNP antibody preparations.

The quantum yield (0.23) of untreated antibody relative to tryptophan is in good agreement with reported values (87). However, in the reduced, alkylated and polyalanylated antibody and isolated heavy chains, the tryptophan fluorescence is markedly enhanced. This enhancement is not due to a decrease in polarity of the local environment around the tryptophan residues, otherwise the  $\lambda_{max}$ of emission would be shifted to lower wavelengths (143,144); actually, a slight shift (341 to 345 mµ) in the opposite direction has been observed. The enhancement of fluorescence could be the result of a conformational change in the RAP-Ab-3 and RAP-Ab-3-H This change may either (a) remove from the local preparations. tryptophan environment neighbouring residues containing, for example, carbonyl groups which act as energy sinks for dissipation of fluorescence energy (145), or (b) protect the tryptophan residues from collisional quenching by solvent (chloride) ions (146). The relative quantum yields in Table XVIII indicate that the extent of this conformational change is greater in the isolated heavy chain (RAP-Ab-3-H) than in the reduced and polyalanylated antibody (RAP-Ab-3). Optical rotatory dispersion studies on the isolated chains from IgG have in fact shown that the chains possess distinct ordered conformations which are quite different from native IgG (147).

#### GENERAL DISCUSSION

The average intrinsic association constants for the reaction of Fab' with hapten (see Table IX, Chapter III) were found to be 53, 8 and 12 per cent higher than those constants determined for reactions involving the corresponding intact antibodies: Ab-1, Ab-2 and Ab-3, respectively. In two instances, McGuigan and Eisen (87) observed affinity constants for Fab' fragments which were 74 and 13 per cent higher than those for intact anti-DNP antibodies; however, in another antibody preparation, these authors found that the affinity constant determined for reactions involving Fab' was 14 per cent lower than that determined for the reaction of the parent antibody with hapten. Apparently these authors did not consider these differences significant.

Since there is no absolute method for measuring the affinity of antibody for hapten and which can be used to distinguish between antibody preparations differing only slightly in affinity, there is a tendency to minimize the significance of any small observed differences in affinity constants. The factor which most severely limits the accuracy of the various techniques for studying the binding of hapten is the heterogeneity of affinity of antibodies for hapten. High affinity anti-DNP antibodies were found to be heterogeneous both with respect to tryptophan content and fluorescence, so that titration with limited amounts of hapten caused disproportionately more quenching of fluorescence than the fraction of sites occupied (87). Moreover, the accuracy of all techniques used in equilibrium studies deteriorates rapidly approaching experimental conditions of hapten excess, when

binding sites of lowest affinity in a heterogeneous population of antibodies are involved in reactions with hapten. As a result, when plotting the experimental data for the determination of equilibrium parameters of stoichiometry, affinity constants and indices of heterogeneity, deviations from linearity are usually observed under these conditions.

In spite of these limitations, the observation made in this study that the affinity for hapten of Fab' fragments from three antibody preparations, as determined by two different techniques and using two different haptens, was slightly higher than that of the parent antibodies, seems to be quite real. Confidence in the observed differences in affinity was strengthened by the consistency in the equilibrium data, since binding studies were performed in duplicate and in some cases in triplicate. Moreover, the binding constants, obtained independently from temperature-jump kinetic data (Table XII), were in fairly good agreement with those obtained from equilibrium data.

The binding constants calculated in the present study confirm the earlier observations that the hapten, lN-2,5S-4DNP, is bound less strongly by anti-DNP antibodies than  $\epsilon$ -DNP-lysine (92). The lower affinity for lN-2,5S-4DNP is most likely due to steric hindrance by the naphthol 5-sulfonate group (92). However, it is to be emphasized that although the affinity constants for the reactions of anti-DNP antibodies or their Fab' fragments with  $\epsilon$ -DNP-lysine were 100 times greater than those for the corresponding reactions with lN-2,5S-4DNP, no significant differences in the association rate constants ( $k_{12}$ ) for the two systems were detected. Thus, one can conclude that differences in affinity constants for different hapten-antibody systems appear to be governed solely by the "lifetime" of the hapten-

antibody complex i.e. by the rate constant, k<sub>21</sub>, of the dissociation step.

For reacting species having spherical symmetry, in the absence of charge effects, the diffusion controlled rate constant may be calculated by the expression (148):

$$k_{12} = \frac{2 \pi N}{1000} \cdot R_{1,2} \cdot D_{1,2}$$
 (19)

where N is Avogadro's number,  $R_{1,2}$  represents the reaction radius, and  $D_{1,2}$  is the sum of the diffusion coefficients of the interacting molecules. Using this equation for the hapten-antibody system, one would predict that diffusion would limit the rate constant to about  $10^9 \text{ M}^{-1} \text{ sec}^{-1}$ , with an apparent activation energy resulting from the temperature dependence of solvent viscosity of 4-5 kcal/mole (95). This value for the rate constant is about 2 orders of magnitude larger than the experimental rate constants obtained in the present study and in earlier investigations on hapten-antibody reactions (listed in Table III, Chapter II). The above rate expression is, of course, an oversimplification since coulombic interactions were neglected. The hapten-antibody reaction, therefore, could well be diffusion controlled, the observed lower association rate constant being a reflection of the rigid steric requirements for binding (149).

While all Fab' fragments had only a slightly higher affinity for a given hapten than the parent intact antibody, the corresponding association rate constants for reactions involving Fab' fragments were almost twice as high as those for reactions involving the antibody molecule. The observed differences in the forward rate constants, assuming a diffusion controlled reaction, cannot be explained by the fact that Fab' is a smaller molecular entity than the antibody molecule and, therefore, diffuses faster. The rate constants, in accordance with equation (19), are proportional to the sum of the diffusion coefficients of both reactants. However, in view of the considerably higher diffusion coefficients of the haptens as compared to those of antibodies or their fragments (i.e. by a factor of the order of 10), the rate constants of the reactions involving the fragments would not be expected to exceed those of reactions involving intact antibodies by more than 10 percent. Therefore, the observed almost two-fold increase in  $k_{12}$  must be attributed to factors other than differences in diffusion coefficients.

To account for the observed higher rate constants of reactions involving Fab' fragments, the following tentative explanation, based on possible differences in accessibility of the antibodycombining sites in the intact antibody molecule and its fragments, is proposed. According to the kinetic theory of collisions (150), the rate constant may be written as

$$k_{12} = PZe^{-Eexp/RT}$$
(20)

where  $E_{exp}$  is the experimental activation energy and Z, the collision number, is identified with the frequency factor of the reaction. The factor P appearing in this expression is referred to as a probability or steric factor and is introduced to take into consideration the fact that not all collisions may be effective in complex formation between the reacting species. If one considers diffusion of the hapten molecule into a hemispherical site, as suggested by Alberty and Hammes for enzyme-substrate reactions (148), then if both antibody-combining

sites were not located on the outer periphery of the Fab units in an intact molecule [Fig. 33a] as they would be by necessity in the separated fragments but were directed toward each other [Fig. 33b], partial screening of the combining site on one Fab unit by the other would reduce the solid angle of approach of the hapten required to produce an effective collision leading to complex formation. This effect of one site shielding the other in the intact molecule would be more pronounced the closer the distance between the two Fab units.

Indirect evidence that the binding sites in the intact molecule are actually oriented toward each other can be derived from the recent study of Warner and Schumaker (151). These authors, on the basis of their centrifugal data, suggested that the two combining sites of an anti-DNP antibody may be linked by the divalent haptens,  $\alpha$ ,  $\varepsilon$ -diDNPlysine or diDNP-cysteine. Considering the relatively small distance between the two dinitrophenyl determinant groups in these molecules, the cross-linking of the two binding sites could take place if indeed they were facing each other.

This interpretation is further supported by the observation that the rate constant  $(k_{12})$  for the reaction between mildly reduced, alkylated and polyalanylated antibody (RAP-Ab-3) and  $\varepsilon$ -DNP-lysine is higher than that for the corresponding reactions with unmodified antibody (Ab-3); in fact, it was almost identical to that calculated for reactions involving the Fab' fragments, Ab-3-Fab' (Table XVII, Chapter IV). The alanine sidechains, estimated to be 6-7 residues long, would prevent the Fab units from approaching each other close enough to reduce the frequency factor of effective collisions, so that the kinetics of reaction of polyalanylated antibody with hapten

# Figure 33

Models of the intact  $\gamma G$  antibody molecule indicating the positions of its combining sites.



would more closely resemble the kinetics of corresponding Fab' reactions. One can conclude, therefore, that the probability distribution of the Fab units with respect to each other in the native antibody molecule is highest at a distance of separation which is significantly less than in the polyalanylated antibody.

In the intact unmodified antibody, the spatial arrangement of the Fab units may be affected by noncovalent interactions between their component heavy chains when the Fab units are in close proximity. The degree of flexibility of the Fab units about the hinge region (Fig. 2) may thus be somewhat restricted, yet the IgG molecule may have more flexibility than would be expected from a globular protein, since the frictional coefficient ratio was found to be 1.47 (48) which is higher than that expected for globular proteins (1.1 - 1.3). Even if there was no interaction between the Fab moieties of an antibody molecule, one would expect these subunits to spend some time in close proximity. The degree of flexibility has been investigated by rotational relaxation studies (49,50,51), but there was quite a disagreement in the reports. The evidence presented here seems to indicate a restricted "amplitude of oscillation' of the Fab units in the intact antibody molecule.

The rate of dissociation of the Fab'-hapten complex was found on the average to be 1.5 times faster than the corresponding rate of dissociation of hapten-antibody complexes. Thus, it can also be postulated that cleavage of an antibody molecule into Fab' fragments results in some slight conformational change in or near the combining sites, making each site somewhat less rigid. An increased rate of dissociation of the Fab'-hapten complex would result if the attractive forces between the hapten and the binding site were

reduced as a consequence of any conformational changes in the site. The increased flexibility of the combining site could also allow a greater number of collisions between the hapten and the site to be effective.

It must be pointed out that the present investigation does not provide any evidence for a conformational change in the intact antibody molecule upon combination with hapten since, as in all previous experiments with a temperature-jump method, only one relaxation effect was observed. However, it can be visualized that if an equilibrium existed between the two forms of an antibody molecule, one of these exhibiting a slight interaction between the Fab regions and the other having non-interacting subunits, complex formation with hapten might lead to a stabilization of the latter. It is suggested that close investigation of the entropy terms and the activation parameters involving intact antibody,  $F(ab')_2$  dimers and Fab' fragments might throw additional light on the problem.

According to the theory of absolute reaction rates (150), the rate constant for a bimolecular reaction in solution is given by the expression:

$$k_{12} = e^2 \frac{\Delta S^*/R}{h} \cdot e \cdot e^{-E} e^{xp/RT}$$
(21)

which enables one to calculate the entropy of activation,  $\Delta S^*$ . In this equation the probability factor P in equation (20) is interpreted in terms of the entropy factor, e  $\Delta S^*/R$ . Regrettably, the temperature dependence of the rate constants was not determined in the present study due to lack of sufficient antibody. However,

further experiments to determine the entropy and energy of activation may provide an unambiguous explanation for the differences im association rate constants for reactions involving Fab' fragments and their parent antibodies, since these differences may represent contributions of  $E_{exp}$  and/or  $\Delta S^*$ . The bivalent  $F(ab')_2$  dimer is included in the suggestion for further studies since it provides an ideal intermediary between the intact antibody molecule and its univalent Fab' fragments. Kinetic investigations with this molecule should help to decide whether the rate of reaction of hapten with antibody is influenced by interactions in the Fc or Fd regions of the heavy chains.

The effects of reduction, alkylation and polyalanylation on antibody activity can be clearly deduced from equilibrium and kinetic data. Equilibrium studies on the antibody preparation, RAP-Ab-3, have shown that the strength of binding of hapten by the antibody site is decreased by a factor of 2-3 relative to the affinity of the Fab' site for hapten. This loss in stability of the hapten-antibody complex resulted in an increased dissociation rate constant (1.80 sec<sup>-1</sup>) compared to that for the Fab'-hapten complex (0.80 sec<sup>-1</sup>). Kinetic data indicate, however, that the binding site in the modified antibody molecule is still relatively intact, since the rate constants  $(k_{12})$  of association with hapten are almost identical for the RAP-AD-3 and Ab-3-Fab' preparations, the value of the rate constant limited only by the rates at which the two reaction partners can diffuse together. It was also shown that 1.6 sites per RAP-Ab-3 antibody molecule (or 0.8 sites per Fab unit) were titratable with hapten. One interpretation of the data is that reduction of the interchain

disulfide bonds causes a loosening of the tertiary structure of the Fab moiety. The increased flexibility of the antibody-combining site could have been the result of conformational adjustments of the polypeptide chains that may have removed one or more of the 'contact' amino acids in the binding site. The 'contact' amino acids are defined as those residues which are directly involved in bonding with the hapten through van der Waals and electrostatic forces, hydrogen bonding etc. Another possibility is that, in an antibody population heterogeneous with respect to amino acid composition and sequence, alanine sidechains may have been formed in some molecules in or near the binding site effectively destroying its antibody activity. This could account for the fact that only 0.8 combining sites were found per Fab subunit of preparation RAP-Ab-3.

This interpretation of the data is supported by the evidence that the maximum quenching of fluorescence (42.5 per cent) by hapten is much lower that that observed for untreated antibody (65.3 per cent) and Fab' fragments (73.4 per cent), owing to the fact that the transfer of fluorescence energy to bound hapten is less efficient the less proper the orientation between the hapten acceptor and the fluorescence donor. A conformational change is also suggested by the enhanced fluorescence of the RAP-Ab-3 preparation ( $Q_r = 0.33$ ) compared to the untreated antibody ( $Q_r = 0.22$ ).

The equilibrium and kinetic data for the reactions of hapten with the isolated polypeptide chains of anti-DNP antibodies provide very interesting information for speculation on the location of the antibody-combining site and the role played by both  $\gamma$  and light chains in forming the combining site. The most popular view (see Chapter I) is that the antibody-combining site resides in the heavy chain,

and that the light chain playes an indirect role, probably by stabilizing the conformation of the site on the heavy chain. As reported in Chapter IV, no antibody activity was detected in the isolated light chains, RAP-Ab-3-L, by the fluorescence quenching technique. Similar results were obtained by Haber and Richards (60) using fluorescence quenching and equilibrium dialysis techniques and Jaton et al (61) using the equilibrium dialysis technique. It is possible that the light chains may contribute directly to the 'contact' amino acids in the binding site, but that the residual affinity for hapten in the isolated light chains may be so weak as to escape detection. This possibility seems hardly likely, however, since Haber and Richards could observe no activity whatscever in IgG molecules reconstituted from specific anti-DNP light chains and heavy chains from normal y-globulin; in contrast, recombination of specific anti-DNP heavy chains with nonspecific light chains produced the same recovery of sites (50 per cent) as with specific light chains, though the binding constant was lower by a factor of 10 (60).

Both rate constants for the reversible reaction between anti-DNP heavy chains (RAP-Ab-3-H) and  $\varepsilon$ -DNP-lysine were found to be much lower than those determined for the reaction of the parent antibody with  $\varepsilon$ -DNP-lysine. The association rate constant,  $k_{12}(3 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1})$ , was about 3 orders of magnitude lower and the dissociation rate constant,  $k_{21}(0.07 \text{ sec}^{-1})$  about 1 order of magnitude lower than the corresponding rate constants for reactions involving the intact antibody. If removal of the light chains had left the portion of the binding site in the heavy chain practically unchanged with respect to conformation and rigidity, then one would expect the rate constant

for association of the heavy chain with hapten to be of the same order of magnitude ( $\sim 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ ) as that found for most hapten-antiboly reactions. The 60-fold reduction in affinity constant would then be reflected in a corresponding increase in the dissociation rate constant, owing to the loose fit of the hapten in the residual binding site. This was found to be the case when anti-DNP antibodies were reacted with a dye-hapten incorporating the smaller p-nitrophenyl haptenic determinant (92). Since the kinetic data reported in Chapter IV do not support this interpretation (in fact the decreased affinity of the heavy chain preparation was found to be due to a much reduced rate constant of association), it can be concluded that the combining site in the heavy chain was not left intact but had increased in flexibility.

Complete removal of the light chains could drastically reduce the extent of stabilization of the conformation of the site in the heavy chain. The isolated heavy chains have been shown by optical rotatory dispersion studies to assume a new conformation quite different from that which they possessed in the intact antibody (147), as is evident also from the enhanced fluorescence ( $Q_r = 0.44$ ) observed in this study. If one accepts the suggestion of Cathou and Haber (77) that the amino acid residues in the binding site are located in at least several widely separated regions of the chains, brought into a close spatial relationship by the molecular conformation, then when the heavy chain assumes its new conformation, at least some of these 'contact' amino acids may be removed from one another. With this model of the heavy chain in mind, the much reduced rate constants for the reaction of RAP-Ab-3-H with <sub>e</sub>-DNP-lysine could be explained in the following ways:

(a) There may be a two-step mechanism as represented by the relationship

$$Ab + H \xrightarrow{k_{12}} AbH \xrightarrow{k_{23}} AbH^*$$
 (22)

The first step is equivalent to the mechanism for hapten-antibody reactions and represents the diffusion controlled reaction between the hapten and the heavy chain. The second step, the rate limiting step, describes a conformational change in the heavy chain in the presence of hapten, by which the removed 'contact' amino acids are brought into a closer spatial relationship with the hapten. It is possible also that only the second step is observable by the fluorescence quenching technique, since the efficiency of energy transfer is dependent on the spatial relationship between the hapten acceptor and the 'contact' amino acid residues in the site.

(b) An alternative mechanism slightly different from the preceding one is that given by the expression

$$Ab + H \xrightarrow{k_{12}} Ab^* + H \xrightarrow{k_{23}} AbH^*$$
 (23)

In this case the second step represents the diffusion controlled reaction between the interacting species. The first step, the rate limiting one, represents an equilibrium among two or more possible states of the heavy chain of which only one, Ab\*, contains the 'contact' amino acids in the right conformation for effective collision.

It must be pointed out that in view of the rigid steric requirements for binding of hapten and the short range nature of the forces involved in hapten-antibody reactions, the effectiveness of the combining site could be drastically diminished by only slight displacements of the 'contact' amino acids from their original positions in the intact site i.e. by minor alterations in conformation.

(c) There is a third possibility that the hapten may be bound by heavy chain dimers. In the saline-phosphate buffer in which these experiments were performed, the heavy chains were found to dimerize at concentrations of 3 mg/ml. Dimerization of the chains could occur in such a way that the degree of accessibility of the site to hapten is reduced due to steric hindrance by amino acid sidechains not directly involved in binding.

There is no evidence presented inothis study for the selection of any one or more of these views over the others, but (a) and (b) would represent the more likely possibilities. Unfortunately, owing to the poor quality of the reaction curves displayed in the kinetic studies of isolated heavy chains, it was not possible to differentiate between the simple one-step mechanism [equation (1)] for hapten-antibody reactions and those represented by equations (22) and (23). If a conformational change in the binding site is necessary before reaction with hapten can take place, one obvious suggestion for further studies would be to investigate the temperature dependence of the rate constants. Conformational rearrangements would require considerably higher activation energies than normally found for hapten-antibody reactions. Optical rotatory dispersion studies of the conformation of specific heavy chains in the presence and absence of hapten may be helpful also in the detection of any recovery of the original conformation of the heavy chain in the intact antibody molecule. However, this structural

adjustment may occur only in the region of the binding site and may not be discernible from the overall spectrum of the heavy chain. The possibility of active polyalanylated heavy chains existing in dimeric form, as proposed in (c), can be examined by the use of the Yphantis equilibrium ultracentrifugation technique (152), which is applicable to investigations of the molecular size of monodisperse and paucidisperse solutions at concentrations ( $200 \mu g/ml$ ) approaching those used in this study. If it is found that the heavy chains exist as dimers, one would still have to answer the question as to whether a single hapten molecule interacts with 'contact' amino acids of one chain or with 'contact' amino acids of both chains.

It should be pointed out that although the heavy chain preparation used in this investigation was somewhat contaminated with light chains (to the extent of 5-6 per cent), it seems unlikely that the equilibrium and kinetic data presented in Chapter IV were due to the interaction of heavy chain-light chain (H-L) recombinants with hapten. Such recombinants would in this case constitute no more than 13 mole per cent. It is estimated that their presence would lower the valence from 0.45 to 0.40 antibody-combining sites per heavy chain and the affinity constant from  $4 \times 10^5$  to about  $1 \times 10^5$  M<sup>-1</sup>. These estimations are based on the assumptions that the H-L recombinant is univalent and has an affinity constant which is one order of magnitude lower than that for the intact antibody (60). Also any appreciable concentration of such recombinants would have given rise to rates of reaction much more similar to those of the intact RAP-Ab-3 preparation.

The much lower dissociation rate constant  $(0.07 \text{ sec}^{-1})$ indicates that the 'lifetime' of the heavy chain-hapten complex is 10 times longer than that of the Fab'-hapten complex. This observation may be taken as an indication that, once the complex has been formed, at least as many 'contact' amino acids as in the native combining site interact with the hapten. Thus, one can further conclude that amino acids of the light chain do not participate directly in the combining site, and that the light chain only confers rigidity to the combining site by "fixing" the spatial relationship of the 'contact' amino acids in the heavy chain. The evidence presented in this study, therefore, supports the findings of Jaton et al (61) that the information required to form the binding site of the heavy chain is contained in the amino acid sequence of the Fd region, with the implication that during antibody biosynthesis the heavy chains may be folded so as to present an antibody-combining site prior to association with light chains to form the antibody molecule.

### SUMMARY

1) The affinity of anti-DNP antibodies and their univalent Fab' fragments for the haptens, 1-hydroxy-4-(2,4-dinitrophenylazo)-2,5-naphthalene disulfonate (1N-2,5S-4DNP) and  $\varepsilon$ -DNP-lysine, was studied by spectrophotometric and fluorescence quenching techniques. In all cases the Fab' fragments were found to have a slightly higher affinity for the haptens than the intact parent antibodies.

2) Rate constants for reactions of intact antibodies and Fab' fragments with the haptens, 1N-2,5S-4DNP and  $\varepsilon$ -DNP-lysine, were determined using the temperature-jump and stopped-flow techniques. The rate constants of the association step for reactions of Fab' fragments with either hapten  $(2 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1})$  were found to be twice as high as those for reactions involving the intact antibodies. It is postulated that these differences were due to a greater accessibility to the hapten of the antibody-combining site in the separated fragments.

3) In order to increase the solubility of isolated heavy chains from anti-DNP antibody in aqueous non-dissociating buffer solutions, the antibodies were polyalanylated after reduction and alkylation of the interchain disulfide bonds.

4) Equilibrium studies were performed on the reactions of mildly reduced, alkylated and polyalanylated but otherwise intact antibody (RAP-Ab-3) and its component heavy and light chains with  $\epsilon$ -DNPlysine using the fluorescence quenching technique. Reduction and polyalanylation was found to cause a 2-fold reduction in affinity constant of the intact antibody. No antibody activity was detected in the specific light chains. The alanylated heavy chains had an affinity

constant of  $4 \ge 10^5 \text{ M}^{-1}$ , 60 times lower than that of the untreated antibody. The valence was estimated at 0.45 binding sites per heavy chain.

(5) Rate measurements of the reactions of RAP-Ab-3 antibody and its component heavy chains (RAP-Ab-3-H) with  $\varepsilon$ -DNPlysine were performed with the aid of the stopped-flow technique. The rate constant of association of RAP-Ab-3 antibody with hapten  $(1.9 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1})$  was found to be almost identical to that for reactions involving the corresponding Fab' fragments. The difference in affinity constants between RAP-Ab-3 and Fab' reactions was reflected in a corresponding difference in their dissociation rate constants.

The association rate constant for the heavy chain-hapten reaction was calculated to be  $3 \times 10^4$  M<sup>-1</sup> sec<sup>-1</sup>, three orders of magnitude lower than that for corresponding reactions involving intact antibody. The dissociation rate constant was calculated to be 0.07 sec<sup>-1</sup>, one order of magnitude lower than that for the dissociation of the hapten-antibody complex. The much lower rate constants for reactions of heavy chains with hapten indicate an increased flexibility of the residual binding site, probably as a result of conformational changes in the isolated heavy chains. The rather low rate constant of dissociation of heavy chain-hapten complex suggests that in all probability the 'contact' amino acids of the combining site of an anti-DNP antibody molecule are located in the heavy chain.

#### CIAIMS TO ORIGINALITY

(1) Average intrinsic association constants for reactions of anti-DNP antibodies and their univalent Fab' fragments with the haptens IN-2,5S-4DNP and  $\varepsilon$ -DNP-lysine were measured using spectrophotometric or spectro-fluorometric techniques. In all cases, the Fab' fragments were found to have a slightly higher affinity for the haptens than the intact parent antibodies.

(2) Plots of log (Relative Fluorescence Intensity) against hapten concentration were used to correct for hapten attenuation and to determine Q<sub>max</sub> values in fluorescence quenching studies.

(3) Kinetic studies of the reactions listed under (1) were performed using the temperature-jump relaxation and stopped-flow techniques. Association rate constants for reactions involving Fab' fragments were found to be almost twice as high as those for reactions involving intact antibodies. These differences in rate constants were interpreted in terms of differences in the accessibility to hapten of the combining sites of Fab' fragments and intact antibody molecules, or in terms of small differences in the conformation of the respective combining sites.

(4) The rate constants for the reaction of reduced, alkylated and polyalanylated but otherwise intact antibody (RAP-Ab-3) with ε-DNP-lysine were calculated from stopped-flow kinetic data. The association rate constant for the RAP-Ab-3: ε-DNP-lysine system was found to be almost identical to that determined for the Fab': ε-DNP-lysine system. Differences in affinity constants were governed solely by differences in the dissociation rate constants.

(5) Rate measurements of the reaction of polyalanylated  $\gamma$  chains with  $\epsilon$ -DNP-lysine were carried out using the stopped-flow technique. The

reaction was found to be juch slower than the corresponding antibody-hapten reaction. The rate constants for the association and dissociation steps were calculated to be  $3 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$  and  $0.07 \text{ sec}^{-1}$ , respectively. The much reduced rate constants for  $\gamma$  chain-hapten reactions were attributed to an increased flexibility of the antibody-combining site, stemming probably from conformational changes in the  $\gamma$ chain. These results indicate that the light chains do not contribute directly to the 'contact' amino acids in the antibody site.

### APPENDIX A

k.

For the one-step mechanism.

$$Ab + H \xrightarrow{12} AbH$$
(1)

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the rate equation governing this mechanism is

$$\frac{d[AbH]}{dt} = k_{12}(Ab)(H) - k_{21}(AbH)$$
(2)

The concentration of the reactants at any time t, i.e. (Ab), (H) and (AbH), may be expressed in terms of the corresponding equilibrium concentrations ( $\overline{Ab}$ ), ( $\overline{H}$ ) and ( $\overline{AbH}$ ) and small deviations from these values. Thus (Ab) = ( $\overline{Ab}$ ) +  $\Delta$  (Ab) (H) = ( $\overline{H}$ ) +  $\Delta$  (H) (3)

$$(AbH) = (AbH) + \Delta (AbH)$$

Substitution into equation (2) yields

$$\frac{d[(\overline{AbH}) + \Delta(AbH)]}{dt} = k_{12} [(\overline{Ab}) + \Delta (Ab)] [(\overline{H}) + \Delta(H)] - k_{21} [(\overline{AbH}) + \Delta(AbH)]$$
$$= k_{12} [(\overline{Ab}) (\overline{H}) + (\overline{Ab}) \Delta (H) + (\overline{H}) \Delta (Ab) + \Delta (H) \Delta (Ab)]$$
$$- k_{21} (\overline{AbH}) - k_{21} \Delta (AbH)$$
(4)

If the deviations from equilibrium are small, the term  $\Delta$  (H)  $\Delta$  (Ab) becomes negligible, and since

$$\Delta (AbH) = -\Delta (Ab) = -\Delta (H)$$
 (5)

 $k_{12}(\overline{Ab})(\overline{H}) = k_{21}(\overline{AbH})$  at equilibrium,

one can write

and

$$\frac{d \Delta(AbH)}{dt} = -\{k_{21} + k_{12}[(\overline{Ab}) + (\overline{H})]\} \Delta(AbH)$$
(6)

Separation of variables gives

$$\frac{d \Delta (AbH)}{\Delta (AbH)} = - \{k_{21} + k_{12} [\overline{(Ab)} + (\overline{H})]\} dt$$
(7)

which can be integrated to

$$\ln \Delta(AbH) = - \{k_{21} + k_{12}[(\overline{Ab}) + (\overline{H})]\}t + I$$
(8)

The constant of integration, I, is found from the boundary condition to be equal to  $\ln \Delta(AbH)_0$ , where  $\Delta(AbH)_0$  is the value of  $\Delta(AbH)$  when t = 0; then

$$\ln \frac{\Delta(ADH)_{o}}{\Delta(ADH)} = \{k_{21} + k_{12} [(\overline{AD}) + (\overline{H})]\} t$$
(9)

The relaxation time  $\tau$  is defined as the time corresponding to

$$\frac{\Delta (AbH)_{o}}{\Delta (AbH)} = e$$
(10)

where e is the base of the natural logarithms; it is therefore the time at which the distance from equilibrium,  $\Delta(AbH)$ , is 1/e of the initial distance,  $\Delta(AbH)_0$ . Since ln e = 1, the relaxation time is seen from equation (9) to be

$$= \frac{1}{k_{21} + k_{12}[(Ab) + (H)]}$$
(11)

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$$\frac{1}{\tau} = k_{21} + k_{12} [(\overline{Ab}) + (\overline{H})]$$
 (12)

### Treatment of Data

The relaxation curves obtained for the Ab-1-Fab' : 1N-2,5S-4DNPsystem, monitored at 520 mµ, represented the increase in complex,  $\Delta(AbH)$ , with time t. The half-times  $(t_{\frac{1}{2}})$  of the reaction were determined from plots of log  $\Delta(AbH)$  against time; relaxation times were then calculated using the relationship

$$= \frac{t_{\frac{1}{2}}}{\ln 2} = \frac{t_{\frac{1}{2}}}{0.693}$$

(13)
•	Т	ab	1	e	XIX
	_		-	-	

$\left[(\overline{Ab}) + (\overline{H})\right] \times 10^5 [M]$	1.26	1.48	1.79	2.26	2.34	2.66
1/r [sec <sup>-1</sup> ]	322	381	408	555	453	533
	315	239	462	495	468	555
	277	347	542	533	578	578
	282	418	542	533	472	578
	330	394	357	513	.533	578
•	347	389	462	502	462	550
	333	305	347	587	506	555
	343	350	347	521	555	630
	398	396	375	550	517	542
	369	347	420	513	533	660
	428	396	375	453	533	603
	282	365	408	542	420	533
	373	385	433	513	495	555
	354	322	462	578	578	559
	462	396	365	<b>49</b> 5	630	693
	277	433	420	533	533	648
	330	498	408	513	630	648
1/τ [sec <sup>-1</sup> ]	342	368	419	525	523	588
Standard Deviation	52	48	60	32	59	50

Relaxation data for the system Ab-1-Fab': 1N-2,5S-4DNP

Let  $\left[\overline{(Ab)} + \overline{(H)}\right] =$ x

and  $1/\tau$ 

 $y = k_{21} + k_{12} x$ 

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=

Then

n

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 $\Sigma x_{i} = 200.43 \times 10^{-5}$ ;  $\Sigma y_{i} = 47,049$  $= 1.97 \times 10^{-5}$ Ā ÿ ;

$$\sum x_{1}^{2} = 418.9 \times 10^{-10} ; \sum y_{1}^{2} = 22,775,089$$

$$\sum (x_{1} - \overline{x})^{2} = \sum x_{1}^{2} - n\overline{x}^{2} = 25.05 \times 10^{-10} ; \sum (y_{1} - \overline{y})^{2} = 1,073,046$$

$$\sum x_{1}y_{1} = 96,976 \times 10^{-5}$$

$$\sum (x_{1} - \overline{x}) (y_{1} - \overline{y}) = \sum x_{1}y_{1} - n\overline{x}\overline{y} = 4,524 \times 10^{-5}$$

$$k_{12} = \frac{\sum (x_{1} - \overline{x}) (y_{1} - \overline{y})}{\sum (x_{1} - \overline{x})^{2}} = \frac{4,524 \times 10^{-5}}{25.05 \times 10^{-10}} = 181 \times 10^{5} \text{ M}^{-1} \text{ sec}$$

$$k_{21} = \overline{y} - k_{12}\overline{x} = 106 \text{ sec}^{-1}$$

The equation of the best fitting line is therefore  $y = 106 + 181 \times 10^5 [(\overline{Ab}) + (\overline{H})]$ 

The sum of the squared deviations,

$$S^2 = \Sigma (y_i - \bar{y})^2 - k_{12} \Sigma (x_i - \bar{x})^2 = 255,873$$
  
The mean square deviation  $= \frac{S^2}{n-2} = 2,559$ 

The variance in  $k_{12}$ ,  $V_{(k_{12})} = \frac{s^2}{n-2} \cdot \frac{1}{\Sigma(x_i - \bar{x})^2} = 102 \times 10^{10}$ 

The variance in 
$$k_{21}$$
,  $V_{(k_{21})} = \frac{s^2}{n-2} \left[ \frac{1}{n} + \frac{\bar{x}^2}{\Sigma(x_i - \bar{x})^2} \right] = 419$ 

The upper  $97\frac{1}{2}$  per cent point of the t distribution with (n-2) degrees of freedom is 1.98. The 95 per cent confidence interval of  $k_{12}$  is therefore

$$k_{12} = 181 \times 10^5 \pm 1.98 \sqrt{V_{(k_{12})}} = 1.81 \pm 0.20 \times 10^7 M^{-1} sec^{-1}$$

Similarly, the 95 per cent confidence interval of  $k_{21}$  is

$$k_{21} = 106 \pm 1.98 \sqrt{v_{(k_{21})}} = 106 \pm 41 \text{ sec}^{-1}$$

#### APPENDIX B

Deviation of the rate equation for the treatment of stopped-flow kinetic data

For the system

the rate of formation of AbH may be expressed as

$$\frac{dc}{dt} = {}^{k}_{12} (a_{o}^{-c})(b_{o}^{-c}) - {}^{k}_{21}c$$
(2)

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(1)

(4)

where a and b are the initial concentrations of Ab and H, respectively, and c is the concentration of AbH at time t. Substitution into (2) for  $k_{21} = k_{12} K_o^d$  yields

$$\frac{dc}{dt} = k_{12} (a_0 - c) (b_0 - c) - k_{12} K_0^d c$$
(3)

where  $K_0^d$  is the average intrinsic dissociation constant. At equilibrium, AbH =  $c_e$ , and

$$\frac{dc}{dt} = \frac{k_{12} (a_o - c_e) (b_o - c_e) - k_{12} K_o^d c_e}{k_{12} [c_e^2 - (a_o + b_o + K_o^d) c_e + a_o b_o] = 0}$$

On solving the quadratic equation for  $c_e$ , one obtains

$$c_{e} = \frac{1}{2} \left[ a_{o}^{+}b_{o}^{+}K_{o}^{d} \pm \sqrt{(b_{o}^{-}a_{o}^{+}K_{o}^{d})^{2} + 4a_{o}^{-}K_{o}^{d}} \right]$$
 (5)

The (+)ve sign is dropped since  $c_e = 0$  when  $a_o = b_o = 0$ 

Therefore 
$$c_e = \frac{1}{2} \left[ a_0 + b_0 + K_0^d - \sqrt{-Q} \right]$$
 (6)  
Where  $\sqrt{-Q} = \sqrt{(b_0 - a_0 + K_0^d)^2 + 4a_0 K_0^d}$  (7)

Wh

The extent of reaction, x, is given by the expression

$$\kappa = \frac{c}{c_{a}}$$
(8)

$$\frac{dc}{dt} = {}^{c}e \frac{dx}{dt}$$

Substitution in equation (3) for dc/dt and c leads to

$$\frac{dx}{dt} = \frac{k_{12}}{c_e} [(a_o - xc_e)(b_o - xc_e) - xc_e K_o^d]$$
(9)

Substitution for  $c_e$  [equation (6)] and rearranging yields finally the rate equation

where 
$$\phi = (\frac{\frac{dx}{dt}}{a_{0} + b_{0} + K_{0}^{d} + \sqrt{-Q}}) (1-x) (1-\phi x)$$
 (10)  
(11)

Separation of the variables gives

$$\frac{dx}{(1-x)(1-\phi x)} = \frac{1}{2} k_{12} (a_0 + b_0 + K_0^d + \sqrt{-Q}) dt$$
(12)

The expression on the left may be integrated by the method of partial fractions; thus equation (12) may be written as

$$\left(\frac{A}{1+x} + \frac{B}{1-\phi_x}\right) dx = \frac{1}{2} k_{12} \left(a_0 + b_0 + K_0^d + \sqrt{-Q}\right) dt$$
(13)

By equating coefficients between the left-hand sides of equations (12) and (13), it is easily found that

$$A = \frac{1}{1-\phi} \quad \text{and} \quad B = \frac{-\phi}{1-\phi} \quad (14)$$

Equation (13) therefore becomes

$$\frac{1}{(1-\phi)} \frac{dx}{1-x} - \frac{\phi dx}{1-\phi x} = \frac{1}{2} k_{12} \left( a_0 + b_0 + K_0^d + \sqrt{-Q} \right) dt$$
(15)

Integration gives

$$\frac{1}{(1-\phi)} \cdot \ln \left(\frac{1-\phi x}{1-x}\right) = \frac{1}{2} k_{12} \left(a_0 + b_0 + K_0^d + \sqrt{-Q}\right) t + I \quad (16)$$

when t=0, x=0, so that the constant of integration I=0 Thus

$$\frac{1}{(1-\phi)} \cdot \frac{\ln (\frac{1-\phi_x}{1-x})}{(1-x)} = \frac{1}{2} k_{12} (a_0 + b_0 + K_0^d + \sqrt{-Q}) t$$
(17)

or

$$\frac{1}{\sqrt{-Q}} \frac{\ln}{(1-x)} = \frac{k}{12}t$$
 (18)

$$\log \left(\frac{1-\phi_x}{1-x}\right) = \frac{k_{12} \sqrt{-Q}}{2.303} t$$
 (19)

## APPENDIX C

The treatment of data for a typical fluorometric titration experiment.

### Table xx

Experimental data for the titration of Ab-3-Fab' (75  $\mu g/ml)$  with  $\epsilon$ -DNP-lysine (1.06 x 10^{-4} M).

Total Volume hapten added m1]0.00000.00760.01520.02280.03440.050Hapten concentration [µM] (H)T0.000.400.801.201.802.60Relative Fluorescence Intensity, RFI96.069.048.837.631.327.9Blank Corrected RFI95.068.047.836.630.326.9Dilution Corrected RFI95.068.348.237.030.827.6Normalized RFI100.071.950.738.932.429.1log RFI2.00001.85681.70461.58941.51011.463log (hapten attenuation)0.00000.00530.01060.01590.02390.034		and the second se	The second s				and the second
Hapten concentration $[\mu M]$ (H) T0.000.400.801.201.802.60Relative Fluorescence Intensity, RFI96.069.048.837.631.327.9Blank Corrected RFI95.068.047.836.630.326.9Dilution Corrected RFI95.068.348.237.030.827.6Normalized RFI100.071.950.738.932.429.1log RFI2.00001.85681.70461.58941.51011.463log (hapten attenuation)0.00000.00530.01060.01590.02390.034Corrected RFI100.072.851.940.334.231.5	Total Volume hapten added [m1]	0.0000	0.0076	0.0152	0.0228	0.0344	0.0502
Relative Fluorescence Intensity, RFI96.069.048.837.631.327.9Blank Corrected RFI95.068.047.836.630.326.9Dilution Corrected RFI95.068.348.237.030.827.6Normalized RFI100.071.950.738.932.429.1log RFI2.00001.85681.70461.58941.51011.463log (hapten attenuation)0.00000.00530.01060.01590.02390.034Corrected RFI100.072.851.940.334.231.5	Hapten concentration [µM] (H) <sub>T</sub>	0.00	0.40	0.80	1.20	1.80	2.60
Blank Corrected RFI95.068.047.836.630.326.9Dilution Corrected RFI95.068.348.237.030.827.6Normalized RFI100.071.950.738.932.429.1log RFI2.00001.85681.70461.58941.51011.463log (hapten attenuation)0.00000.00530.01060.01590.02390.034Corrected RFI100.072.851.940.334.231.5	Relative Fluorescence Intensity, RFI	96.0	69.0	48.8	37.6	31.3	27.9
Dilution Corrected RFI95.068.348.237.030.827.6Normalized RFI100.071.950.738.932.429.1log RFI2.00001.85681.70461.58941.51011.463log (hapten attenuation)0.00000.00530.01060.01590.02390.034Corrected RFI100.072.851.940.334.231.5	Blank Corrected RFI	95.0	68.0	47.8	36.6	30.3	26.9
Normalized RFI100.071.950.738.932.429.1log RFI2.00001.85681.70461.58941.51011.463log (hapten attenuation)0.00000.00530.01060.01590.02390.034Corrected RFI100.072.851.940.334.231.5	Dilution Corrected RFI	95.0	68.3	48.2	37.0	30.8	27.6
log RFI2.00001.85681.70461.58941.51011.463log (hapten attenuation)0.00000.00530.01060.01590.02390.034Corrected RFI100.072.851.940.334.231.5	Normalized RFI	100.0	71.9	50.7	38.9	32.4	29.1
log (hapten attenuation)0.00000.00530.01060.01590.02390.034Corrected RFI100.072.851.940.334.231.5	log RFI	2,0000	1.8568	1.7046	1.5894	1.5101	1.4638
Corrected RFI 100.0 72.8 51.9 40.3 34.2 31.5	log (hapten attenuation)	0.0000	0.0053	0.0106	0.0159	0.0239	0.0345
	Corrected RFI	100.0	72.8	51.9	40.3	34.2	31.5

Sample Calculation:

The hapten concentration, on addition of 0.0152 ml of  $1.06 \times 10^{-4}$  M  $\epsilon$ -DNP-lysine to 2 ml of protein solution,

$$(H)_{\rm T} = (\frac{0.0152}{10^3} \times 1.06 \times 10^{-4}) \times (\frac{10^3}{2.0152}) \times 10^6$$

$$=$$
 0.80  $\mu$ M.

Blank Corrected RFI = Observed RFI of sample - RFI of solvent

Dilution Corrected RFI =  $47.8 \times \frac{2.0152}{2.00} = 48.2 \%$ 

Normalized RFI =  $48.2 \times \frac{100.0}{95.0} = 50.7 \%$ 

The hapten attenuation coefficient was estimated to be 0.0133  $\log(%T)/\mu M$  of hapten (see Chapter III). Hapten attenuation correction:

> log (normalized RFI) + hapten attenuation = 1.7046 + 0.0133 x 0.80 = 1.7152

Corrected RFI = antilog 1.7152 = 51.9 %T

The corrected RFI was then plotted as a function of hapten concentration,  $(H)_{T}$ , to yield the specific quenching curve (Fig. 15).

# APPENDIX D

In Chapter III, the total concentration of antibody sites,  $[Fab]_{T}$ , in Ab-1-Fab' preparations was determined by extrapolation of the specific binding curve (see Fig. 13 and Table VI). Since the average intrinsic association constant,  $K_{o}^{a} = 1/c$  when  $1/b = 2 \times 1/[Fab]_{T}$ the estimated  $K_{o}^{a}$  value is therefore dependent on the extrapolated value of  $1/b[=1/[Fab]_{T}]$  at 1/c = 0. From the triplicate binding experiments using different concentrations of Ab-1-Fab', the concentration of sites in the stock Ab-1-Fab' solution was calculated to be  $4.05 \pm 0.24 \times 10^{-5}$ M, and  $K_{o}^{a}$  was estimated at  $2.30 \pm 0.62 \times 10^{5}$  M<sup>-1</sup>. The 6% error in estimation of the total concentration of sites, therefore, resulted in about a 30% deviation in  $K_{o}^{a}$ .

The 6% error in the determination of total binding site concentration by this method was verified by precipitin analysis (Kjeldahl nitrogen determination) on the whole antibody preparation, Ab-1. Also, the value of the equilibrium constant  $(1.7 \times 10^5 \text{ M}^{-1})$ obtained independently by the temperature-jump technique from the ratio  $k_{12}/k_{21}$  fell within the range of 2.30 ± 0.62 × 10<sup>5</sup> M<sup>-1</sup> found for the Ab-1-Fab': 1N-2,5S-4DNP system.

Similar calculations of the errors involved in fluorescence binding studies showed a deviation of about 20% in  $K_0^a$  values. It is possible, therefore, from these deviations in  $K_0^a$  values that the reactions of intact antibody and their Fab' fragments with hapten are indistinguishable by their affinity constants.

Calculation on the kinetic data show, however, that these deviations in antibody site concentration and  $K_0^a$  values are not

sufficient to produce a factor of 2 in the association rate constants for reactions involving intact antibody and its Fab' fragments. In the temperature-jump experiments, for instance, if  $k_{12}$  was the same for both systems ( $1.80 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ ), then on substituting in the rate equation:

$$l/\tau = k_{21} + k_{12} [(\overline{Ab}) + (\overline{H})]$$

for  $1/\tau = 300 \text{ sec}^{-1}$  and  $k_{21}$  (average) = 90 sec<sup>-1</sup>, one obtains  $[(\overline{Ab}) + (\overline{H})] = 1.17 \times 10^{-5} \text{ M}.$ 

For the Ab-1: 1N-2,5S-4DNP system, when  $1/\tau = 300 \text{ sec}^{-1}$ 

 $(\overline{H}) = 0.43 \times 10^{-5} M$   $(\overline{Ab}) = (1.17 - 0.43) \times 10^{-5} = 0.74 \times 10^{-5} M$   $(Ab)_{bound} = (H)_{bound} = 0.71 \times 10^{-5} M$   $(Ab)_{Total} = (\overline{Ab}) + (Ab)_{bound} = 1.45 \times 10^{-5} M$ measured  $(Ab)_{Total} = 2.46 \times 10^{-5} M$ 

So that the % error in the estimation of total binding site concentration required to equate  $k_{12}$  values

$$= (\frac{2.46 - 1.45}{1.45}) \times 100 = 70\%$$
,

far in excess of the estimated experimental error.

It was found also that antibody site concentration and  $K_o^d$   $(=1/K_o^a)$  values were not critical in the estimation of  $k_{12}$  values from stopped-flow kinetic data. Thus, deviations of 50% in antibody site concentrations produced only about 10% changes in  $k_{12}$  values. The precise value of  $K_o^d$  is not a critical factor since the reverse reaction is of no great consequence in the extents of reaction measured. The largest uncertainty appears to be the rate measurements themselves which were shown in Table XII to yield rate constants accurate to within  $\pm 15\%$ .

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