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EQUILIBRIUM AND KINETIC STUDIES ON THE REACTION OF ANTIBODY WITH A UNIVALENT ANTIGEN

BY

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A univalent antigen, DNPI, was prepared by coupling a 2,4-dinitrophenyl (DNP) group onto the sole lysyl residue at position 29 of the B chain of bovine insulin. Spectrophotometric analysis revealed that only one DNP group had been introduced per insulin dimer. Column and thin-layer gel filtration chromatography experiments in 0.01M PBS, pH 7.4 established that the DNPI existed as dimers (M.W. = 12,000) at a concentration of 1×10^{-4}M.

The affinity constant (K_o^a) for the reaction of anti-DNP antibodies and their univalent Fab' fragments with DNPI, as determined by fluorescence quenching, was found to be about one half of that for the corresponding reaction involving the hapten ε -DNP-lysine. The binding of DNP-Insulin by both intact antibody and Fab' fragments appeared to be more homogeneous than the binding of ε-DNP-lysine by the same antibody preparation.

Kinetic experiments performed with the stopped-flow technique revealed that both bivalent antibody and Fab' fragments reacted with DNP-Insulin in a "biphasic" manner: an initial rapid drop-off in fluorescence was followed by a slower approach to equilibrium. Therefore, the following mechanism was proposed:

$$Ab + Ag \rightleftharpoons AbAg^* \rightleftharpoons AbAg$$

where the second step represents a rearrangement of the complex. Rate constants of association for the initial reaction of DNPI with intact antibody and their univalent Fab' fragments were found to be $4.7 \times 10^6 M^{-1} sec^{-1}$ and $5.3 \times 10^6 M^{-1} sec^{-1}$, respectively. Both rate constants were lower than those observed by others for reactions involving DNP-lysine.

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Chapter I

THE PROPERTIES OF ANTIGENS AND ANTIBODIES

INTRODUCTION

Immunity is the principal defensive system of higher organisms against disease, leaving the host in a higher state of resistance after the initial attack. This immune phenomenon has been observed by mankind since the time of Thucydides. The Chinese have, since the fifteenth century, practised prophylactic induction of smallpox infection by inhalation of dried powders of smallpox crusts through the nose 'in the same manner as we take snuff' (Humphrey and White, 1970).

Beginning in the 15th century, this awareness led to deliberate attempts to study the nature of immunity. The picture became somewhat clearer, when in 1888 Roux and Yersin first demonstrated that antibody was formed during the process of immunization(Humphrey and White, 1970). Subsequently, a large number of investigators turned their attention to the fundamental aspects of the nature in which antibody interacted.

with its homologous antigen. Paul Ehrlich was the first to believe that the antigen-antibody interaction is fundamentally a chemical reaction. This realization served dramatically to open the way for chemists and physists, as well as immunologists to study immunity.

PROPERTIES OF ANTIGENS

An antigen is a substance which, when introduced into an animal or human body, is capable of eliciting the production of specific antibodies. To be immunogenic, an antigen must be a) foreign to the host into which it is injected, and b) of a minimum size, usually greater than M.W. 10,000; however, it has been demonstrated recently that substances with as low a molecular weight as 450 are immunogenic (Borek et al., 1965, 1967), furthermore, the unconjugated glucagon (3485 mol. wt.) (Worobec et al., 1972) and angiotensin I (1436 mol. wt.) and II (1158 mol. wt.) (Worobec et al., 1972a) have also been shown able to elicit specific antibodies. Immunogenicity is a property not of the whole macromolecule, or microorganism, but of certain molecular segments of unique configuration known as antigenic determinants. Thus, it was shown by Landsteiner (1945) that antibodies could be elicited by some small,chemically well defined molecules, called haptens, if these were coupled to macromolecular carriers. Haptens are not immunogenic as such, but will react with antibodies formed to hapten-protein conjugates.

PROPERTIES OF ANTIBODIES

Antibodies are immunoglobulins which have the property of reacting specifically with and binding to the antigenic determinants

of the antigen which has been used for the immunization.

Currently, five classes of human immunoglobulins are known. designated as IgM, IgG, IgA, IgD and IgE (Table I). These are differentiated on the basis of their antigenic properties and/or structural differences in their heavy chains. The fact that immunoglobulin (IgG) consists of four polypeptide chains (two heavy and two light) was first demonstrated by Edelman et al. (1961, 1963) and Fleischman et al. (1962). They were able to show this by reduction of the protein with mercaptoethanol, followed by separation of the chains by starch gel electrophoresis in 8 M urea, or gel chromatography in propionic acid. The molecular weight of a single light chain is about 22,500, and that of a heavy chain is about 50,000 (Figure 1).

Porter (1959) subjected rabbit IgG antibodies to papain digestion in the presence of cysteine. The antibodies were degraded into three large fragments of approximately equal molecular weight. One of these fragments called Fc had no affinity for the antigen and was shown to be crystallizable. The other two fragments were very similar to one another, each possessing a single combining site. These were called Fab fragments (Figure 1). It was later demonstrated (Nisonoff et al., 1960) that pepsin would split IgG to produce divalent $F(ab')_2$ fragments, which, when treated with thiol reagents, would yield two univalent Fab' subunits.

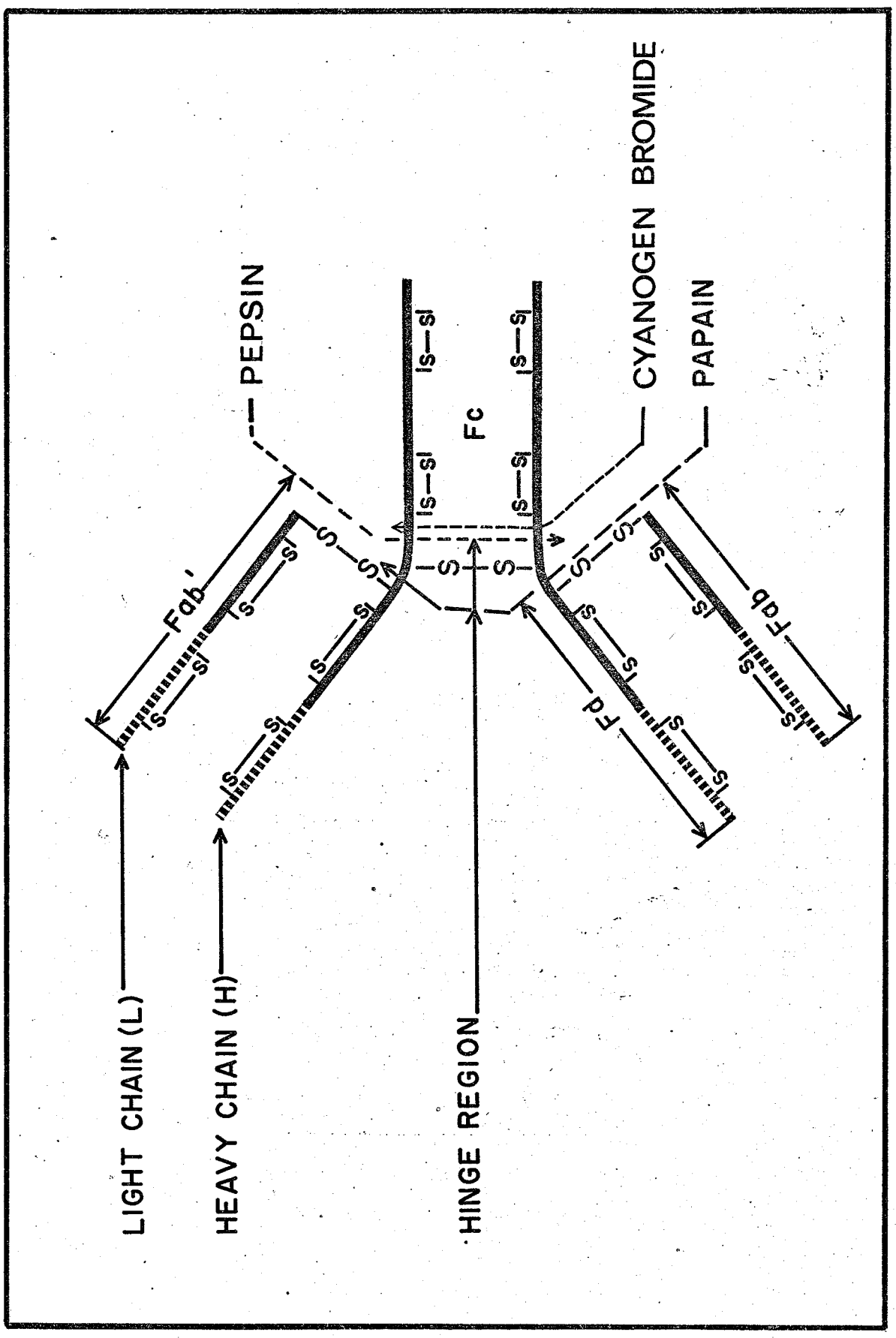
Antibody Combining Site

One of the fundamental problems in immunochemistry is to understand the structural basis of antibody specificity and variability. The antibody combining sites are located in the N-terminal half of the

TABLE I

CLASSES OF HUMAN IMMUNOGLOBULINS

Characteristics	Class				
	IgG	IgA	IgM	IgD	IgE
Molecular weight	150,000	170,000 to 500,000	900,000	200,000	200,000
Heavy chains:					
Class	γ	α	μ	δ	ε
Subclasses	$\gamma_{1,2,3,4}$	$\alpha_{1,2}$	$\mu_{1,2}$		
Molecular weight	53,000	63,000	75,000	77,000	75,000
Allotypes	Gm	Am	-	-	-
Light chains:					
Class	κ λ	κ λ	κ λ	κ λ	κ λ
Molecular weight	22,500	22,500	22,500	22,500	22,500
Allotypes	Inv	Inv	Inv	Inv	Inv
Molecular formula	$(\kappa_2\gamma_2)$ or $(\lambda_2\gamma_2)$	$(\kappa_2\alpha_2)_n$ or $(\lambda_2\alpha_2)_n$ $n = 1,2,3$	$(\kappa_2\mu_2)_5$ or $(\lambda_2\mu_2)_5$	$(\kappa_2\delta_2)$ or $(\lambda_2\delta_2)$	$(\kappa_2\varepsilon_2)$ or $(\lambda_2\varepsilon_2)$
Antigen binding sites	2	2	5-10	2	2
$S_{20,w}$	6.5-7.0	7,10,13, 15	18-20	6.2-6.8	10.7
Carbohydrates (%)	2.9	7.5	11.8	14.8	10.7
Serum levels (mg/%)	600-1600	20-500	60-200	0.1-40	0.01-0.9
Complement fixing	+	0	+		0
Skin sensitizing	heterologous species	-	-	-	+


PEPSIN
CYANOGEN BROMIDE
PAPAIN
Fab'
Fc
Fd
Fab
LIGHT CHAIN (L)
HEAVY CHAIN (H)
HINGE REGION

Fab fragments, which are composed of the light chain and Fd region of the heavy chain.

The number of unique antibody combining sites has been estimated to be not less than 10^6 (Eisen, 1968; Haurowitz, 1970). Two general hypotheses have been proposed to explain the structural basis of this large number of antibody specificities. The first was advanced by Breinl and Haurowitz (1930), Haurowitz (1957) and Pauling (1940), and postulated that all antibody molecules consist of identical polypeptide chains which can be folded into different conformations. Recent evidence has shown that this is not the case. Instead, Fab' fragments have been shown to regain their specificity when unfolding by denaturing reagents is followed by renaturation (Haber, 1964; Whitney and Tanford, 1965). This evidence, as well as the discovery that heavy (Press and Piggot, 1967; Gottlieb et al., 1968; Wang et al., 1971) and light (Hilschmann and Craig, 1965; Milstein, 1966; Titani et al., 1966) chains have regions of variable amino acid sequence at the N-terminal end, gave much support to the second hypothesis which states that different antibody specificities are a reflection of different primary structures.

Although it has been established that both heavy and light chains have variable regions, more difficulty has been experienced in establishing whether one or both chains contribute contact amino acids to the combining sites. Most direct methods such as hapten binding implicated heavy chain contribution (Metzger and Singer, 1963; Givol and Sela, 1964; Haber and Richards, 1966; Kelly, 1970), although some weak binding by light chains has also been observed (Roholt et al., 1963; Yoo et al., 1967; Parker and Osterland, 1970; Painter et al.,

1972; 1972a). Similarly, when heavy chains of one specificity were mixed with light chains of another specificity to form a reconstituted antibody molecule, this reconstituted antibody usually possessed the specificity of the heavy chain, although light chains of the same antibody preparation more effectively restored this specificity than light chains of other antibodies (Haber and Richards, 1966; Jaton et al., 1968; Painter et al., 1972; 1972a).

Affinity labelling experiments have always implicated both chains as partners in the antibody combining site: Singer et al. (1966) studying anti-DNP antibodies observed labelling in both chains with a heavy/light chain ratio of 2:1. Fleet et al. (1969) and Press et al. (1971) using the nitro-phenyl azo (NAP) group and corresponding antibodies observed a ratio of 3.5:1.0. Conclusions that both chains contributed to the combining site were also made by Weinstein et al. (1969) in a study of an anti-DNP myeloma protein with two affinity labels of different size.

Published data reporting upon the size of an antigenic site have been largely obtained with a series of linear antigens, including the dextrans (Kabat, 1960) and polyalanyl — as well as polylysyl — polypeptides (Sage et al., 1964; Schechter and Sela, 1965). Present indications are that an antibody site can encompass at least as many as six to seven isomaltose residues, or four to five amino acid residues. The dimensions of the combining site for anti-carbohydrate antibodies have been estimated to be 34 x 12 x 7 Å (Kabat, 1970). Using electron spin resonance (ESR), Hsia and Little (1971) arrived at the conclusion that the combining site of rabbit anti-TNP antibody has a longitudinal dimension of 10 Å.

Heterogeneity of Combining Sites

The heterogeneity of antibody combining sites in a given antibody population is reflected in many ways. Thus, Nisonoff and Pressman (1958), Eisen and Siskind (1964), Little and Eisen (1966) and Pressman and Grossberg (1968) were able to show heterogeneous binding of hapten by its homologous antibody. Moreover, heterogeneity of electrophoretic mobility was observed when light chains from specifically purified antibody preparations were analyzed by disc electrophoresis (Choules and Singles, 1966). Other evidence for Ab heterogeneity was observed in the variety of amino acid sequences on the N-terminal end of light chain from rabbit anti-DNP antibody (Doolittle, 1966); in the different tryptophan content at the proximity of the site (McGuigan and Eisen, 1968); and finally in the diversity of the antigenic specificities of idiotypic antibodies (Oudin and Michel, 1969; McDonald and Nisonoff, 1970; Nisonoff et al., 1972).

It has been speculated that the source for this combining site heterogeneity arises in two different ways: the first may be due to the immunological heterogeneity of antigens. The multiple antigenic determinants on the commonly used protein antigens would obviously elicit many antobodies with different specificities.

Antibodies elicited by a wide variety of simple haptenic groups conjugated with protein carriers also display molecular heterogeneity (Karush, 1962). The cause of this heterogeneity has been demonstrated to arise mainly from the carrier effect in the immune response. The hapten itself may be only a part of the immunogenic determinant group, which in addition may contain the adjacent

areas of the surface of the protein carrier. Hence, the specific antibodies thus formed will also recognize part of the amino acid residues from the protein carrier (Parker et al., 1966; Benacerraf et al., 1966, 1970; Windelhake and Voss, 1970). In addition, the hapten molecules may be bound through different amino acid residues in the protein, or, as Singer (1964) and his associates (Reid et al., 1971) have shown that even though a hapten molecule is attached to a fixed amino acid residue on a carrier, the hapten molecule may non-covalently fix to other amino acid residues depending on whether it resides in a hydrophylic or hydrophobic environment which, potentially affects the antigenic properties of this hapten-protein conjugates. Perhaps, the foregoing can be applied to explain the heterogeneity of anti-DNP antibodies in immunization of animals with mono-DNP-RNase (Eisen et al., 1964a), or with mono-DNP-Insulin (Little and Counts, 1969).

Secondly, the combining site heterogeneity of antibodies could be elicited even by relatively homogeneous immunogenic determinants, capable of stimulating clones of cells, which in turn produce similar, but not identical antibodies.

It is generally believed now that the heterogeneity of antibodies to haptens is not best described by a continuous Gaussian distribution, but consists of a limited number of antibody populations each having uniform binding constants, and each being the product of a single clone of cells (Pressman et al., 1970).

It follows from the preceding discussion that antibody derived from a single committed cell should structurally and functionally exhibit the properties of homogeneous antibody. Indeed, it has

recently been discovered that several myeloma proteins, each of which is produced by an individual plasma cell tumor, have uniform primary structure (Potter et al., 1964) and homogeneous ligand binding activity (Eisen et al., 1970). In vivo, a single anti-DNP antibody-producing cell clone can be propagated in syngeneic mice (Askonas et al., 1970), and its anti-DNP antibody shows homogeneous properties (Montgomery et al., 1972).

Anti-hapten antibodies of restricted heterogeneity have been elicited with DNP-protein antigens in adult rabbits (Roholt et al., 1970) and in higher yield in neonatal rabbits (Montgomery and Williamson, 1970). Anti-DNP antibody with restricted isoelectric spectrum and a homogeneous binding constant ($\alpha = 1.0$) has been produced in rabbits by hyperimmunization with $(DNP)_2$-Gramicidin-S as antigen (Montgomery et al., 1972). Using the isoelectric focusing technique, Freedman et al. (1972) were able to isolate a fraction of homogeneous anti-DNP antibody that was elicited by immunizing with a conventional DNP-protein antigen. Antibodies with a homogeneous binding constant have also been elicited by the polypeptide hormones angiotensin I and II (Worobec et al., 1972a, 1972b). In addition, bradykinin (Haber et al., 1967), vasopressin (Wu and Rockey, 1969) and some bacterial polysaccharides (Haber, 1970; Eichmann et al., 1970) have also been used to induce structurally homogeneous antibodies.

MANIFESTATION OF ANTIBODY-ANTIGEN REACTIONS IN VITRO

The Precipitin Reaction

When increasing amounts of an antigen are added to a constant amount of antiserum a precipitate is formed; the amount increases at

first, reaches a maximum at the equivalence zone, and then decreases again in antigen excess. The precipitin curve so obtained was originally explained qualitatively by the frame-work theory of Pauling (1940). The assumption made at that time was that antibody and antigen were at least divalent. More recent studies have born this out fully.

Immunodiffusion

This technique, which in its most refined form was developed by Ouchterlony (1949), is based on the same principle as the precipitin curve. Antigen and antibody are allowed to diffuse towards one another in a medium of agar. When optimum relative concentrations of antibody and antigen are present, a precipitin line forms. This technique is particularly useful for more complex antigen-antibody systems. The number of lines of precipitate formed correspond to the minimum number of antigens present in a complex mixture of antigens. Quantities of antibody as low as 5-10 μg can be detected.

Immunoelectrophoresis

In principle, this method, developed by Grabar and Williams (1953), is an extension of double diffusion in gel. The various antigens of a complex mixture are separated according to charge by electrophoresis in a medium of agar. Subsequent diffusion against an appropriate antiserum reveals a number of precipitin arcs, which is usually greater than that observed by analysis according to Ouchterlony.

Passive Hemagglutination

This technique also makes use of the aggregation between antigen and antibody, the antigen is either adsorbed to red cells treated with tannic acid (Boyden, 1951) or it is coupled to these

cells by stable covalent bonds using a bifunctional reagent such as bis-diazotized benzidine (BDB) (Stavitsky and Arquilla, 1955; Gordon et al., 1958). An addition of antibody results in an agglutination or clumping of the 'sensitized' red cells. Minute amounts (about 0.03 μg/ml) of antibody can be detected by this method.

Chapter II

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

HAPTEN-ANTIBODY REACTIONS

Equilibrium Studies

If antibody combining sites on a single IgG molecule are considered equal and independent of each other, the hapten-antibody reaction can be represented by the equation:

$$Ab + H \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AbH \qquad (1)$$

where k_{12} and k_{21} are the rate constants of the forward and reverse reactions, respectively.

From the law of mass action and the relation in (1), the equilibrium constant K for this one-step reaction can be written as

$$K = \frac{(AbH)}{(Ab)(H)} = \frac{k_{12}}{k_{21}} \qquad (2)$$

Studies on the bimolecular reaction between a hapten and its specific antibody have contributed greatly to the understanding of more complex antigen-antibody reactions. The complexity of the latter is due mainly to the fact that most antigens are multi-valent and multi-determinant and thus combine with antibody to form aggregates, some large enough to precipitate. Such inhomogeneous equilibria are difficult to treat so that hapten-antibody reactions have served as model systems instead.

Several relatively simple techniques are available for the study of hapten-antibody reactions.

(a) Equilibrium Dialysis

This method has been extensively used for estimating the equilibrium constants of hapten-antibody reactions since it is both thermodynamically sound and easy to perform. The hapten and antibody solutions are placed in two separate chambers, separated by a cellulose membrane which is freely permeable to the hapten molecule but impermeable to the large antibody molecule. At equilibrium the concentration of free hapten is identical in both antibody and hapten compartments and is determined by spectrophotometric measurements (Eisen and Karush, 1949) or by measurements of radioactivity (Nisonoff and Pressman, 1958). Equilibrium dialysis is the most reliable method of measuring hapten binding and is frequently used as a reference method.

(b) Spectrophotometric Method

Certain azo-dyes that incorporate a hapten molecule undergo spectral shifts when combining with specific antibody. This property has proven particularly useful for kinetic studies since the rate

of hapten-antibody reactions could be followed directly (Froese, 1968; Kelly et al., 1971). The hapten molecule is usually coupled through an azo group to a naphthol sulfonate. Binding to antibody normally results in a pK shift of the naphtholic -OH group. Thus, the reaction is pH dependent.

Binding is usually determined by optical density measurement at one or two wavelengths (Metzger et al., 1963a). The latter method is generally more precise since it does not require an accurate knowledge of the amount of hapten added. The concentration of bound and free dye-hapten can be calculated with the aid of the equations:

$$b = \frac{\varepsilon_{f1} O.D._2 - \varepsilon_{f2} O.D._1}{\varepsilon_{f1}\,\varepsilon_{b2} - \varepsilon_{f2}\,\varepsilon_{b1}}$$

$$c = \frac{\varepsilon_{b2} O.D._1 - \varepsilon_{b1} O.D._2}{\varepsilon_{f1}\,\varepsilon_{b2} - \varepsilon_{f2}\,\varepsilon_{b1}}$$

where b = concentration of bound hapten

c = concentration of free hapten

ε_f = extinction coefficient of free hapten

ε_b = extinction coefficient of bound hapten

1 and 2 denote the two different wavelengths used

This method has a disadvantage over equilibrium dialysis since the special dye-haptens required are frequently not available commercially. It is, however, less tedious to perform as the antibody can be titrated directly with the hapten.

(c) Fluorescence Quenching

First applied to hapten-antibody reaction studies by Velick et al. (1960), this method makes use of the fact that the tryptophan fluorescence of the antibody, produced by excitation at 280 mμ , is quenched by haptens which absorb near 350 m μ. This method is thus ideally suited for the 2,4-dinitrophenyl (DNP) hapten-antibody system. It is extremely sensitive, allowing the titration of very dilute antibody solutions. Results obtained by this method frequently have to be checked by a less unambiguous method, such as equilibrium dialysis (Eisen and McGuigan, 1971).

Other methods for studying hapten-antibody binding are based on the fluorescence properties of the hapten, such as fluorescence enhancement (Yoo et al., 1967) and fluorescence polarization (Levison et al., 1971).

Treatment of Binding Data

This is based on the law of mass action as expressed by equation (2). Modification and rearrangement of this equation leads to

$$\frac{r}{c} = Kn - Kr \qquad (3)$$

where c is the free hapten concentration; r, the ratio of hapten bound to total antibody concentration; and n is the antibody valence. A plot of r/c versus r should yield a straight line with the slope equal to K, the equilibrium constant. However, in practice a linear relationship is seldom observed, since, as pointed out in the preceding chapter, most antibody populations are heterogeneous. Nisonoff and Pressman (1958, 1958a) modified equation (3) according to an equation originally derived by Sips (1948);

$$\frac{r}{n} = 1 + \frac{1}{(K_o c)^{\alpha}} \qquad (4)$$

where α (values from 0 to 1) is the heterogeneity index, and K_o is the average intrinsic association constant. It is obvious that when $\alpha = 1$, equation (4) is identical to equation (3), and thus the antibody population is homogeneous, since equation (3) is based on the assumption that all binding sites with respect to K are identical. The smaller the α value, the greater the degree of heterogeneity. In many instances another equation, which can be derived from (4) is used:

$$\frac{1}{b} = \frac{1}{(K_o c)^{\alpha}\ (Ab)} + \frac{1}{(Ab)} \qquad (5)$$

where b is the concentration of bound hapten and Ab is the total concentration of antibody combining sites. However, if both K_o and α are to be determined, it is best to use the equation

$$\log \left(\frac{r}{1 - r}\right) = \alpha \log c + \alpha \log K_o \qquad (6)$$

which is a logarithmic form of (4).

Once the equilibrium constant K_o is known, other thermodynamic parameters may be derived from the following equations:

$$\Delta F^{o} = -RT \ln K_o \qquad (7)$$

$$\frac{d \ln K_o}{d(1/T)} = -\frac{\Delta H^{o}}{R} \qquad (8)$$

$$\Delta F^{o} = \Delta H^{o} - T \Delta S^{o} \qquad (9)$$

where R is the gas constant, T is the absolute temperature and ΔF°, ΔH° and ΔS° are the standard free energy, enthalpy and entropy of reaction, respectively.

Kinetic Studies

Although kinetic studies are quite commonly used to elucidate the mechanisms of chemical reactions, it was only a few years ago that the first attempts were made to apply the methods of chemical kinetics to hapten-antibody reactions. Sehon and Schneider (1961) using cathode-ray polarography observed that the hapten-antibody reaction was complete within the time of mixing of the reactants, i.e., 1 - 2 seconds. Coupled with studies on the rate of dissociation of hapten-antibody complexes, they were able to obtain lowest limiting rate constant of 1×10^{6} $M^{-1}sec^{-1}$ and 1 sec^{-1} for the forward and reverse reactions, respectively. Similar limiting values for the forward step were calculated by Sturtevant et al. (1961) from stopped-flow data.

Thus, it became obvious that conventional techniques of chemical kinetics were not applicable to the hapten-antibody reaction, particularly since in a diffusion controlled reaction the upper limit for the forward reaction could be expected to be as high as 1×10^{9} M^{-1} sec^{-1}. As a consequence, only the stopped-flow method of Hartridge and Roughton (1923) and the temperature-jump relaxation technique of Eigen and DeMaeyer (1962) have been applied successfully to hapten-antibody reactions.

(a) The Stopped-Flow Method

The reactants are placed into separate syringes and are then expelled simultaneously and rapidly through a mixing jet into a reaction chamber. The flow is stopped by filling a third syringe which makes contact with a 'stop' and triggers a recording system (usually an oscilloscope) at the same time. In principle, any optical property of the hapten or antibody can be used as a means of following the reaction. However, because the hapten-antibody reactions are extremely fast, low concentrations of the order of 10^{-6} to 10^{-7} M must be used, so that the half-times of reaction are significantly greater than the dead-time of mixing. Thus, in practice, only methods involving changes in the fluorescence properties of either the antibody or the hapten can be used. Day et al. (1963) and Kelly et al. (1971) made use of fluorescence quenching to study the reactions between anti-DNP antibodies and ε-DNP-lysine. Levison et al. (1971) made use of fluorescence polarization to study the reaction between fluorescein and anti-fluorescein antibody.

(b) The Temperature-Jump Relaxation Method

Froese et al. (1962) first applied this method to the study of hapten-antibody reactions. Unlike the stopped-flow method, there is no instrumental dead-time of mixing involved, it therefore can be applied to a rather wide range of reactant concentrations. The application of this method to hapten-antibody reactions has been recently reviewed (Froese and Sehon, 1971). The reactants are first allowed to come to equilibrium, and this equilibrium is

then suddenly perturbed by discharging a condenser through the solution, and thus raising the temperature by about 6°C. Re-equilibration to the new equilibrium conditions is then followed by measuring changes in either light absorption (Froese, 1968; Kelly et al., 1971) or tryptophan fluorescence (Pecht et al., 1972). From the appropriate oscilloscope tracings, the relaxation time(s), τ, is (are) calculated. From the concentration dependence of the relaxation time(s), the mechanism for each complex multistep reaction can be elucidated.

Studies involving the reaction of the dye-hapten: 1-hydroxy-4-(2,4-dinitrophenylazo)-2,5 naphthalene disulfonate (1N-2,5S-4DNP) with either intact anti-DNP antibodies or the corresponding Fab' fragments, revealed that τ showed the same, simple concentration dependence for both reactions:

$$\frac{1}{\tau} = k_{21} + k_{12}\,[(\overline{Ab}) + (\overline{H})] \qquad (10)$$

where $(\overline{Ab})$ and $(\overline{H})$ are the free equilibrium concentrations of antibody combining sites and hapten, respectively. Thus, reactions of hapten with either bivalent antibody or univalent Fab' fragments obey the simple mechanism represented by Eq. (1).

ANTIGEN-ANTIBODY REACTIONS

Equilibrium Studies

As pointed out above, antigen-antibody reactions are far more complicated than hapten-antibody reactions. Since proteins

are multivalent and multideterminant, combination of such antigens with antibodies results in the formation of large antigen-antibody aggregates of varying size, complexity and solubility. Precipitation is almost inevitable, and thus will introduce a new and heterogeneous equilibrium system. Moreover, precipitation drives the reaction further towards completion; therefore, the thermodynamic data obtained for these antigen-antibody reactions are not for true homogeneous equilibrium states.

Nevertheless, many experiments have been performed to investigate this complicated system. Singer and Campbell (1955) used ultracentrifugation and electrophoresis to study the BSA and anti-BSA antibody reaction; Talmage (1960) used the Farr half-saturation $(NH_4)_2SO_4$ technique to study the reaction of radioactive BSA with anti-BSA antibody; and Berson and Yalow (1959) used ^{131}I-Insulin to study the binding of this labelled antigen to anti-insulin antibodies obtained from insulin resistant patients. Thermodynamic data were also obtained by studying the binding of an insoluble antigen: Rh(c) positive red cells with its specific anti-Rh(c) antibody (Hughes-Jones et al.,1962). Recently Worobec et al. reported that anti-glucagon (1972) and anti-angiotensin (1972a) antibodies have been elicited in rabbits and the binding activity of this partially purified IgG with radioactive antigens have been studied (Table II). In order to avoid problems associated with precipitation, investigations were carried out under conditions of antigen excess, or at low concentrations using radioactive antigens.

Similarly, in order to avoid the problems due to the multivalency of antigen, several univalent protein antigens had been prepared and their immunogenicity as well as the interactions with specific antibodies were studied. Pepe and Singer (1959) prepared the univalent antigen BSA-S-benzenearsonic acid by reacting N-(p-benzenearsonic acid) iodoacetamide with the -SH group of bovine serum mercaptalbumin. The binding of this antigen by anti-benzenearsonic acid antibodies was studied. Brenneman and Singer (1968, 1970) prepared the hapten-protein antigen (Papain-S-DNPL) by the attaching a single DNP-lysine haptenic group to the unique -SH group in the active site of the papain molecule. This hapten-protein antigen was found to be immunogenic in rabbits. The equilibrium constant for the binding of Papain-S-DNPL to anti-DNP-BGG antibodies was found to be 2×10^6 M^{-1}. Similar studies were performed with DNP-Insulin, a conjugate prepared by coupling 2,4-dinitrobenzene sulfonate to the only lysine residue of insulin located at position 29 of the B chain (Little and Counts, 1969).

Kinetic Studies

Early studies on the rate of antigen-antibody reactions by light scattering (Goldberg and Campbell, 1951; Johnson and Ottewill, 1954) did not yield data which could be used to calculate rate constants nor to postulate reaction mechanisms. Rates of reaction were slow and the reactions observed almost certainly involved aggregate formation, which may have been, to a large extent, not specific. As in the case of equilibrium studies, attempts were made to overcome problems associated with aggregation and precipitation, by performing

experiments at very low concentrations of reactants. For example, Talmage (1960) used antigen (^{131}I-BSA) concentrations as low as 10^{-8}M to study the BSA and anti-BSA antibodies reaction by the Farr technique (1958, 1971). The reactions were stopped at various time intervals, and the concentrations of bound antigen were determined. Rate constants in the range of 10^{5} M^{-1} sec^{-1} have been obtained by this technique. Berson and Yalow (1959) used an even lower concentration of ^{131}I-Insulin (10^{-9}M) to investigate the insulin and anti-insulin system. They were able to separate the labelled antigen in its free and bound forms by electrophoresis, and calculated forward rate constants of the order of 10^{4} to 10^{6} M^{-1} sec^{-1}.

Tsuji et al. (1960, 1963) used pseudo-first order conditions to study the reaction between luciferase and anti-luciferase. After the reaction had gone to completion, the amount of unneutralized luciferase was determined through spectrophotometric measurement of the blue luminescence produced by oxidation of luciferin by molecular oxygen, catalyzed by the luciferase.

The bacteriophage neutralization has also been used as a technique to study the affinity and reaction kinetics of anti-hapten antibody with hapten-bacteriophage conjugates (Karush, 1970). The reaction involved the neutralization by anti-hapten antibody of the infectivity of bacteriophage which has been extensively conjugated with hapten. The neutralization of bacteriophage by antibodies is thought to result from blocking of the site on the bacteriophage which is responsible for its adsorption to the bacterium (Adams, 1959). First-order kinetics were usually obtained for the neutralization reaction

(Adams, 1959). Using DNP-bacteriophage ØX174 and rabbit anti-DNP antibody, Hornick and Karush (1969, 1972) were able to obtain the average association constant (K) = 1 x 10^{11} M^{-1}, the average association rate constant (k_{12}) = 3.7 x 10^{7} M^{-1} sec^{-1}, and the average dissociation rate constant (k_{21}) = 3.4 x 10^{-4} sec^{-1}.

Insoluble antigens such as red cells have been used by Hughes-Jones et al (1962), to study the thermodynamics and kinetics of the reactions with its specific antibody. Purified anti-Rh(c) antibody was labelled with ^{131}I and was then reacted with the Rh(c) positive red cells. The forward association rate constant for this reaction is 2.6 - 3.2 x 10^{4} M^{-1} sec^{-1}, and the dissociation rate constant is 2.2 - 17 x 10^{-4} sec^{-1}.

Kinetic studies of the reaction between the peptide antigen: angiotensin and its homologous antibodies have been performed by Worobec et al (1972b), using the half saturated $(NH_4)_2SO_4$ technique of Farr (1958). The forward rate constants varied from 10^{2} to 10^{6} M^{-1} sec^{-1} for primary and secondary response antibodies, respectively. However, great care must be taken in the interpretation of these results. This is partly due to the reasons that have been given in the preceding section, and partly due to the fact that these techniques cannot be accurately employed to study fast reaction such as antigen-antibody reactions.

Using a stopped-flow technique and following changes in fluorescence polarization, Dandliker and his co-workers (Levison et al, 1971) studied the kinetics of the fluorescein and anti-fluorescein system, the forward rate constant which has been obtained is of the order of 10^{8} M^{-1} sec^{-1}. In earlier work, this group, by employing

only the fluorescence polarization technique to study the kinetics of various antigen-antibody systems either under pseudo-first order conditions or by the method of initial slopes, was able to show that in the initial stages of the reaction the empirical rate law is

$$\text{rate} = k_{12}\,(Ab)(Ag)$$

where (Ag) and (Ab) are the initial antigen and antibody concentration, respectively (Dandliker and Levison, 1967; Levison et al., 1970).

Discussion of Equilibrium and Kinetic Data

(1) As one examines the values of the association constant (K_o) for various hapten-antibody systems in Table II, most of them fall in the order of 10^5 - 10^7 M^{-1}. Higher orders of K_o have been found for some anti-DNP and anti-TNP antibodies, and exceptionally high affinity constants ($\simeq 10^{11} M^{-1}$) have been observed for reactions involving anti-fluorescein antibodies and one preparation of anti-DNP antibodies. Generalization of these results may not be meaningful, since the experimental conditions varied from one to another and since it is well known that antibody affinity varies with the dose of injected immunogen and the length of immunization the animals (Eisen and Siskind, 1964; Walker and Siskind, 1968).

Large variations in K_o values have also been observed for antigen-antibody reactions. It would appear that the earlier data obtained mainly in antigen excess by such techniques as ultra-centrifugation, light scattering and electrophoresis are consistantly lower than more recent results which were obtained quite frequently with univalent antigens and/or more sensitive techniques.

The values of free energy of reaction, ΔF^{o}, also vary to some extent (5 - 12 kcal $mole^{-1}$). These small ΔF^{o} values are proof of the weak bond formation in hapten-antibody reactions (Singer, 1965).

Most conflicting data are observed in the ΔS^{o} values. In the hapten-antibody reactions, ΔS^{o} values varied from -42.8 to + 22 e.u.. In the past, positive ΔS^{o} values have been thought to be due to the release of water in the process of formation of hydrophobic bonds between hapten molecule and antibody as proposed by Kauzmann (1959). However, Karush (1952) postulated that this positive entropy might also be the result of molecular rearrangement within the antibody molecule to a more flexible conformation. A more lucid explanation for the occurrence of positive ΔS^{o} in case of enzyme-substrate complex formation was given by Casey and Laidler (1950). They considered two possibilities: (1) During complex formation, H_2O molecules (bound by the proteins in the regions of charged groups) are released to the liquid state resulting in an increase in translational and rotational degrees of freedom more than sufficient to compensate for the decrease in the degrees of freedom of the components of the complex. (2) If either partner of the complex were forced to undergo some disorganization in structure (perhaps involving the breaking of hydrogen bonds elsewhere in the molecule) to provide complementariness of fit at the site of reaction, an increase in entropy could result.

On the whole, it appears that many of the thermodynamic parameters are either conflicting or still unexplainable. This, as Sehon and Haurowitz (1971) pointed out, should warrant a systematic re-investigation the thermodynamic parameters of the hapten-antibody reactions.

TABLE II

THERMODYNAMIC PARAMETERS FOR HAPTEN-ANTIBODY AND ANTIGEN-ANTIBODY REACTIONS

Antibody specific to	Hapten or Antigen	K_o (M^{-1})	$-\Delta F^o$ (kcal. $mole^{-1}$)	$-\Delta H^o$ (kcal. $mole^{-1}$)	ΔS^o (e.u.)	T (^oC)	Reference
	Hapten						
p-azobenzoate	p-iodobenzoate	4.1×10^4	6.3	4.1	7.3	25	1
	p-(p'-hydroxybenzene-azo)-benzoate	6.0×10^4	6.1	-	-	5	2
p-azobenzenearsonate	Terephthalanilide diarsonate	3.0×10^5	7.4	-1 ± 2	22 ± 9	23	3
	p-(p'-hydroxybenzene-azo)-benzenearsonate	3.5×10^5	7.7	-	-	29	4
d-phenyl-(p-azobenzoylamino) acetate	d-phenyl[p-(p-dimethylamino benzeneazo) benzoylamino] acetate	3.1×10^5	7.5	7.3	1	25	6
p-azobenzene-β-lactoside	p-(p-dimethylaminobenzeneazo) benzene-β-lactoside	1.6×10^5	7.1	9.7	-8.7	25	7
2,4-dinitrophenyllysyl	ε-N-2,4-dinitrophenyl lysine (i)	2.0×10^8	11.3	8.6	9.0	26	5
	(ii)	2.3×10^5	6.8	1.6	17	5	8
	(iii)	2.3×10^7	10.89	19.6	-30.4	12.8	9

TABLE II (Continued 1)

THERMODYNAMIC PARAMETERS FOR HAPTEN-ANTIBODY AND ANTIGEN-ANTIBODY REACTIONS

Antibody specific to	Hapten or Antigen	K_o (M^{-1})	$-\Delta F^o$ (kcal. $mole^{-1}$)	$-\Delta H^o$ (kcal. $mole^{-1}$)	ΔS^o (e.u.)	T (^oC)	Reference
2,4-dinitrophenyllysyl	ε-N-2,4-dinitrophenyl lysine (iv)	1.6×10^8	12	23	-35	25	10
	(v)	2.2×10^7	-	-	-	25	31
	(vi)	1.9×10^6	7.2	-	-	21	12
	2,4-dinitroaniline	2.2×10^6	8.8	16	-26	5.4	9
	2-(2,4-dinitrophenyl-azo)-1-naphthol-3,6 disulfonic acid	9.9×10^7	-	-	-	25	11
2,4,6-trinitrophenyl lysyl	ε-trinitrophenyl lysine	1.4×10^8	11.28	-	-	30	24
	Trinitrophenyl amino caproate	1.5×10^8	11.3	24.35	-42.8	30	24
	2,4-dinitrophenyl aminocaproate	9.3×10^5	8.27	9.43	-3.8	30	24
Fluorescein	Fluorescein	6×10^{10}	-	-	-	22	14

TABLE II (Continued 2)

THERMODYNAMIC PARAMETERS FOR HAPTEN-ANTIBODY AND ANTIGEN-ANTIBODY REACTIONS

Antibody specific to	Hapten or Antigen	K_o (M^{-1})	$-\Delta F^o$ (kcal. $mole^{-1}$)	$-\Delta H^o$ (kcal. $mole^{-1}$)	ΔS^o (e.u.)	T (^oC)	Reference
2,4,6-trinitrophenyl guanosyl	N-trinitrophenyllysine	9×10^6	-	-	-	4	13
	N-trinitrophenyl guanosine	5×10^6	-	-	-	4	13
	5'-monophosphate guanosine	2.3×10^4	-	-	-	4	13
Carboxymethylmorphine	Morphine	3.1×10^6	-	-	-		25
	Ethyl morphine	3.4×10^7	-	-	-		25
Tetrahydrocannabinol	Tetrahydrocannabinol azobenzoic acid	5.7×10^6	-	-	-		30
	Protein antigen						
Bovine serum albumin	Bovine serum albumin (i)	8×10^3	4.9	0 ± 2	18 ± 8	0	15
	(ii)	1.7×10^8	-	-	-	±0.5	16
p-azobenzenearsonate	Bovine serumealbumin-p-azobenzenearsonate	2×10^3	4.5	0 ± 2	17 ± 7	25	17

TABLE II (Continued 3)

THERMODYNAMIC PARAMETERS FOR HAPTEN-ANTIBODY AND ANTIGEN-ANTIBODY REACTIONS

Antibody specific to	Hapten or Antigen		K_o (M^{-1})	$-\Delta F^o$ (kcal. $mole^{-1}$)	$-\Delta H^o$ (kcal. $mole^{-1}$)	ΔS^o (e.u.)	T (^oC)	Reference
p-azobenzenearsonate	Bovine serum mercaptalbumin-S-(p-acetamido-benzenearsonate)		1×10^4	5.0	-	-	0	17
Ovalbumin	Ovalbumin	(i)	1.2×10^4	5.1	0 ± 2	19 ± 8	0	18
		(ii)	2.4×10^8	-	-	-		16
Rh(c)	Rh(c) positive red cells		$0.19 - 1.8 \times 10^8$	10.1	1.5 ± 0.6	28	37	19
Creatine ATP transphosphyorylase	Creatine ATP transphosphyorylase		5×10^8	11.8	0	40	30	20
2,4-dinitrophenyllysyl	DNP-ϕx 174	(i)	1.1×10^{11}	-	-	-	37	21
		(ii)	3.7×10^{10}	-	-	-	25	28
	Papain-S-dinitrophenyllysine		2×10^6	-	-	-		22
2,4-dinitrophenyllysyl (BGG)	DNP-Insulin		5.9×10^7	-	-	-	30	29
2,4-dinitrophenyllysyl (Insulin)	DNP-Insulin		$7.8 \times 10^6 - 2.1 \times 10^7$	-	-	-	30	29

TABLE II (Continued 4)

THERMODYNAMIC PARAMETERS FOR HAPTEN-ANTIBODY AND ANTIGEN-ANTIBODY REACTIONS

Antibody specific to	Hapten or Antigen	K_o (M^{-1})	$-\Delta F^o$ (kcal. $mole^{-1}$)	$-\Delta H^o$ (kcal. $mole^{-1}$)	ΔS^o (e.u.)	T (^oC)	Reference
Glucagon	Glucagon	6.3×10^6 - 3.2×10^9	-	3.8 - 4.7	14-28	4-15	23
Insulin	Insulin	1.0×10^9	15.4	3.6	38	37	26
Angiotensin I	Angiotensin I	4.1×10^6 - 4.3×10^9	9-13	3.9 - 5.2	17.1 - 28.5	25	27
Angiotensin II	Angiotensin II	1.6×10^6 - 4.1×10^8	8.4-12	3.7 - 5.7	14.3 - 25.2	25	27

Ref.:
1) Pressman et al. (1961)
2) Nisonoff and Pressman (1958a)
3) Epstein et al. (1956)
4) Eisen and Karush (1949)
5) Velick et al. (1960)
6) Karush (1956)
7) Karush (1957)
8) Carsten and Eisen (1955)
9) Eisen and Siskind (1964)
10) Barisas et al. (1971)
11) Day et al. (1963)
12) Walker and Siskind (1968)
13) Winkelhake and Voss (1970)
14) Portmann et al. (1971)
15) Singer and Campbell (1955)
16) Levison et al. (1968)
17) Pepe and Singer (1959)
18) Singer and Campbell (1955a)
19) Hughes-Jones et al. (1962)
20) Samuels (1963)
21) Karush (1970)
22) Brenneman and Singer (1970)
23) Worobec et al. (1972)
24) Little and Eisen (1966)
25) Leute et al. (1972)
26) Berson and Yalow (1959)
27) Worobec et al. (1972a)
28) Hornick and Karush (1972)
29) Little and Counts (1969)
30) Grant et al. (1972)
31) Kelly et al. (1971)

(2) An inspection of Table III reveals immediately a striking feature of the kinetics of hapten-antibody reactions. Whereas rate constants k_{12} for the forward reaction are relatively constant (10^7 - 10^8 M^{-1} sec^{-1}) i.e., within about one order of magnitude, much larger variations have been observed for the rate constant of dissociation (10^{-3} - 10^3 sec^{-1}) and it would thus seem that the equilibrium constant, K_o, is determined mainly by the latter. The fact that k_{12} approaches the value for diffusion controlled reactions by 1 to 2 orders of magnitude (Day et al., 1963; Kelly, 1970) and the reaction is characterized by a low energy (4 kcal/mole) of activation (Day et al., 1963), would indicate that the antibody combining site is rather rigid and does not have to undergo a conformational change before reacting with the hapten.

Clear-cut evidence for a conformational change associated with the hapten-antibody reaction has not been obtained from kinetic studies. In two temperature-jump relaxation experiments, a second relaxation effect was observed in each case (Froese, 1968; Froese et al., 1969) However, because of the small amplitude of this effect, its significance could not be established. Some indication of a conformational change as a result of hapten-antibody interaction has been obtained by Warner et al. (1970) on the basis of ultracentrifugal studies. Altered susceptibility to enzymatic degradation of antibodies as result of their combination with hapten has been observed by Grossberg et al. (1965) for IgG, and by Ashman and Metzger (1971) for IgM. However, most of this evidence for the occurrence of a conformational change is not considered to be conclusive (Eisen and Pressman, 1971). Finally,

it should be mentioned that even though most kinetic data for hapten-antibody reactions have been obtained with rather heterogenous antibody preparations, they agree remarkedly well with those involving the IgA myeloma protein MOPC 315 (Pecht et al., 1972).

An analysis of the kinetic data for antigen-antibody reaction indicates that both rate constants of association (k_{12}) and dissociation (k_{21}) are significantly lower than the corresponding rate constants for hapten-antibody reactions. A rigorous comparison, however, is difficult to make since most antigens are multivalent and it would seem that the majority of kinetic data have been computed using molar concentrations of antigen and antibody molecules rather than molar concentrations of antigenic determinants and antibody combining sites. Antigen-antibody reactions could be expected to have lower rate constants of association than hapten-antibody reactions. There are two possible explanations: (1) If these reactions are diffusion controlled, then the large antigen molecule would diffuse much less rapidly than a hapten molecule and thus collide less frequently with the antibody combining sites; (2) The steric hindrance due to amino acid residues not directly involved in either the combining site or the antigenic determinant could be appreciable greater in the case of antigen-antibody reactions. Similarly, lower rate constants of dissociation could be expected for antigen-antibody reactions, if (1) both antibody combining sites react with antigenic determinants on the same molecule, and (2) secondary interactions take place between amino acid residues not directly involved in the active sites of either antigen or antibody. Lastly, it should be pointed out that some of

the methods such as the method of initial slope (Dandliker and Levison, 1967) or phage neutralization, which have had to be used to study the kinetics of the reaction between multivalent antigens and divalent antibodies, may not be very suitable for the detection of the finer points of the reaction mechanism, for example, the rearrangement of the antigen-antibody complex or conformational change involving antibody and/or antigen.

TABLE III

RATE CONSTANTS FOR HAPTEN-ANTIBODY AND ANTIGEN-ANTIBODY REACTIONS

Antibody specific to	Hapten or Antigen	k_{12} ($M^{-1}sec^{-1}$)	k_{21} (sec^{-1})	Ko (M^{-1})	T (^{o}C)	Reference
	Hapten					
2,4-dinitrophenyl lysyl	ε-N-2,4-dinitrophenyl lysine (i)	8×10^{7}	1[b]	7.6×10^{7}	25	1
	(ii)	$1.1 \pm 0.2 \times 10^{7}$	0.51[b]	2.2×10^{7}	25	2
	(iii)	1.3×10^{8}	53	2.5×10^{6}[c]	25	3
2,4-dinitrophenyl lysyl[a]	1-hydroxy-4-(2,4-dinitrophenyl-azo)-2,5-naphthalene disul-fonate (i)	$0.95 \pm 0.13 \times 10^{7}$	76 ± 36[b]	1.3×10^{5}	25	2
	(ii)	1.6×10^{7}	80	2.0×10^{5}	23	4
	1-hydroxy-4-(4-nitrophenylazo)-2,5-naphthalene disulfonic acid	1.4×10^{7}	410	3.4×10^{4}	23	4
	2-(2,4-dinitrophenylazo)-1-naph-thol-3,6-disulfonic acid	8×10^{7}	1.4[b]	9.9×10^{7}	25	1
2,4-dinitrophenyl lysyl[a]	ε-N-2,4-dinitrophenyl glycine	1.9×10^{8}	1.3×10^{3}	1.5×10^{5}[c]	25	3
m-nitrophenyl	4,5-dihydroxy-3-(p-nitrophenyl-azo)-2,7-naphthalene disulfonic acid	1.8×10^{8}	760	2.3×10^{5}[c]	25	5
p-azobenzene arsonate	1-naphthol-4,4-(4'azobenzene azo) phenylarsonic acid	2.0×10^{7}	50	4.0×10^{4}[c]	25	17

TABLE III (Continued 1)

RATE CONSTANTS FOR HAPTEN-ANTIBODY AND ANTIGEN-ANTIBODY REACTIONS

Antibody specific to	Hapten or Antigen	k_{12} ($M^{-1}sec^{-1}$)	k_{21} (sec^{-1})	Ko (M^{-1})	T (^{o}C)	Reference
p-azobenzene arsonate	p-(p-dimethylaminophenylazo)-benzene arsonic acid	1.1×10^{7}	1.4×10^{-3}	7.8×10^{9} [c]	25	6
Fluorescein	Fluorescein	4×10^{8}	$\approx 5 \times 10^{-3}$	$6.5 \pm 1.6 \times 10^{10}$	18	7
	Protein Antigen					
Insulin	Insulin	$10^{4} - 10^{6}$	$10^{-5} - 10^{-3}$	1×10^{9}	37	8
Ovalbumin	Ovalbumin	2×10^{5}	10^{-3}	2×10^{8} [c]		9
	Fluorescein-ovalbumin	4.8×10^{5}	2×10^{-3}	2.4×10^{8}	1.5	10
Bovine serum albumin	Bovine serum albumin	$\approx 10^{5}$	$\approx 10^{-5}$	6×10^{8}	37	11
	Dansyl-bovine serum albumin	3.4×10^{5}	2×10^{-3}	1.7×10^{8}	1.5	10
Rh(c)	Rh(c) red cells	$2.6\text{-}7.4 \times 10^{4}$	$2.2\text{-}17 \times 10^{-4}$ [b]	$\approx 10^{8}$	37	12
2,4-dinitrophenyl	2,4-dinitrophenyl-ϕx174 (i)	3.7×10^{7}	4.3×10^{-4}	1.1×10^{11}	37	13
	(ii)	6.0×10^{6}	3.3×10^{-4}	3.7×10^{10}	25	16
Creatine ATP transphosphorylase	Creatine ATP transphosphorylase	$1.5\text{-}3.0 \times 10^{5}$	-	5×10^{8}	35	14

TABLE III (Continued 2)

RATE CONSTANTS FOR HAPTEN-ANTIBODY AND ANTIGEN-ANTIBODY REACTIONS

Antibody specific to	Hapten or Antigen	k_{12} ($M^{-1}sec^{-1}$)	k_{21} (sec^{-1})	Ko (M^{-1})	T (°C)	Reference
Angiotensin I	Angiotensin I	4.9×10^{2} - 1.8×10^{6}	6.0×10^{-5} - -4.2×10^{-4}	4.1×10^{6} - -4.3×10^{9}	25	15
Angiotensin II	Angiotensin II	6.7×10^{2} - 4.6×10^{6}	9.3×10^{-6} - -4.2×10^{-4}	1.6×10^{6} - -4.1×10^{8}	25	15

a - Antibody is MOPC 315, monomeric IgA.

b - Calculated from $k_{21} = \frac{k_{12}}{Ko}$

c - Calculated from $Ko = \frac{k_{12}}{k_{21}}$

Ref.:

1) Day et al. (1963)
2) Kelly et al. (1971)
3) Pecht et al. (1972)
4) Froese (1968)
5) Froese and Sehon (1964)
6) Ferber (1965)
7) Levison et al. (1971)
8) Berson and Yalow (1959)
9) Dandliker and Levison (1967)
10) Levison et al. (1970)
11) Talmage (1960)
12) Hughes-Jones et al. (1962)
13) Karush (1970)
14) Samuels (1963)
15) Worobec et al (1972a)
16) Hornick and Karush (1972)
17) Froese et al. (1962)

Chapter III

EQUILIBRIUM AND KINETIC STUDIES OF ANTIBODY AND FAB' FRAGMENTS WITH THE UNIVALENT ANTIGEN ε-DNP-INSULIN

INTRODUCTION

As has been pointed out in the preceding chapter, equilibrium and kinetic studies of antigen-antibody reactions are subject to considerable complications, brought about by the multivalency of most antigens. Thus, in order to avoid problems due to aggregation or even precipitation, reactions have to be carried out in very dilute solutions or in antigen excess. Even under these conditions other inherent difficulties remain. The number of antigenic determinants for most natural antigens is not known precisely and, moreover, these determinants are most likely not identical. In addition, even at dilute concentration, calculations of rate constants, for example, have to be based on initial rates, since later stages of the reaction may still be complicated by aggregation.

In the present study it was therefore decided to use a univalent antigen and its homologous antibodies. Such a system would not be complicated by aggregation, and kinetic data obtained for it could be compared readily with those for hapten-antibody systems. Moreover, it was also hoped that such studies might reveal whether the reaction of a univalent antigen with an antibody can be represented by the simple mechanism:

$$Ab + Ag \rightleftharpoons AbAg \qquad (11)$$

or, is better described by the relationship:

$$Ab + Ag \overset{I}{\rightleftharpoons} AbAg^* \overset{II}{\rightleftharpoons} AbAg \qquad (12)$$

where the second reaction step represents a rearrangement of the complex, involving, perhaps, not only amino acid residues of the combining site and the antigenic determinant, but also some interactions of amino acid residues not directly involved in the combining site, and which are brought into close proximity as a result of specific complex formation. Finally, it was hoped to confirm with an antigen-antibody system, the data of Kelly et al. (1971), who had demonstrated that the rate constant of association is faster for a reaction of hapten with Fab' fragment than for the corresponding reaction with the bivalent parent antibody molecule.

As pointed out in the preceding chapter, several authors have used univalent antigens to perform equilibrium studies on their reactions with specific antibodies and/or to test their effectiveness as immunogens. Thus, BSA-S-benzenearsonic acid had been used by Pepe and Singer (1959) to study the interaction with antibodies

specific to benzenearsonic acid; ε -41-DNP-ribonuclease, a univalent antigen made by attaching a DNP group to the lysyl residue at position 41 of ribonuclease, was tested as an immunogen (Eisen et al., 1964a) and Papain-S-DNPL (Brenneman and Singer, 1968, 1970) and DNP-Insulin (Little and Counts, 1969) have been used both as immunogens and as antigens in reactions with anti-DNP antibodies.

In the present study, DNP-Insulin (DNPI) was chosen as a univalent antigen for several reasons:

(a) Insulin is a simple molecule, the primary (Ryle et al., 1955) as well as the tertiary and quaternary (Blundell et al., 1971) structures of which are known (Figure 2).

(b) The insulin molecule contains only one lysyl residue (position 29 of the B chain) and therefore only one DNP group couples with insulin (Little and Counts, 1969).

(c) The lysyl residue is freely accessible as determined by X-ray crystallography (Blundell et al., 1971) (Figure 2b).

(d) The insulin molecule lacks tryptophan and thus does not fluoresce significantly, thereby making DNPI quite suitable for the quenching of antibody fluorescence. Therefore, the equilibrium and kinetic parameters for the system consisting of DNPI and anti-DNP antibodies can be determined with relative ease by fluorescence measurements.

MATERIALS AND METHODS

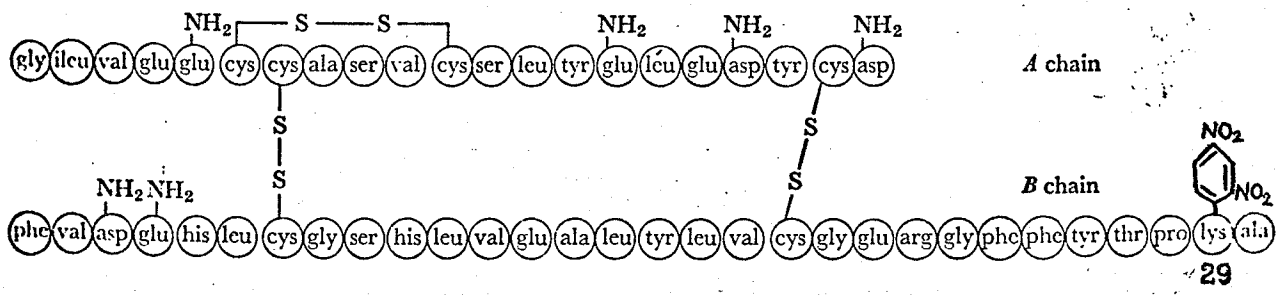
Preparation of ε-DNP-Insulin

The procedure for the preparation of ε-DNP-Insulin (DNPI) was essentially that of Little and Counts (1969). In a typical

FIGURE 2 Structure of Insulin:

(a) Amino acid sequence of bovine DNP-insulin, showing the location of the 2,4-dinitrophenyl lysyl residue.

(b) Atomic positions in the insulin molecules, obtained from X-ray crystallography. Two molecules viewed from different axis. From: Blundell et al., Nature, 231, 506 (1971).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NH2
NH2
NH2
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A chain
S
S
S
S
NO2
NO2
NH2 NH2
B chain
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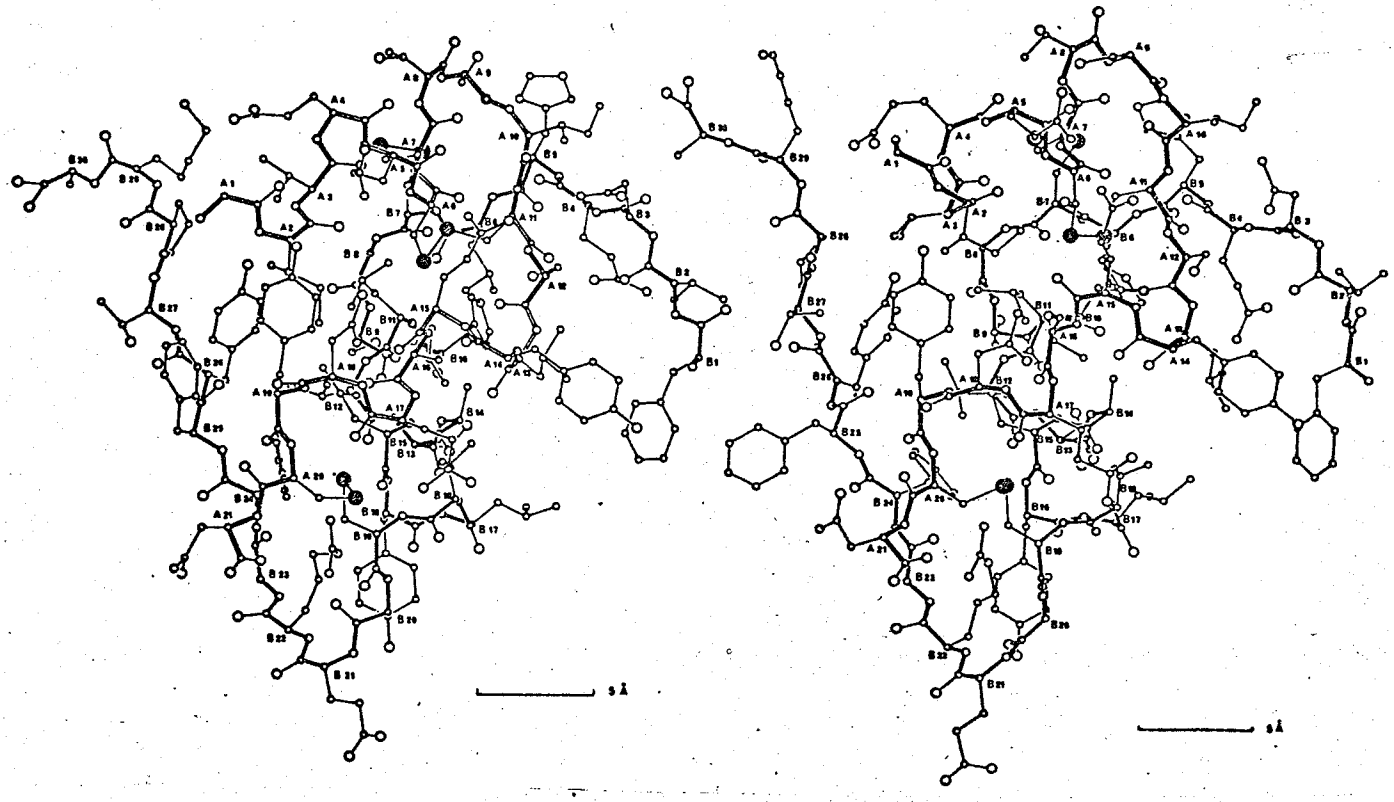
29

(a)



(b)

preparation, 200 mg of bovine crystalline insulin (Connaught Medical Research Laboratories, Toronto; or Sigma Chemical Co., St. Louis) were dissolved in 35 ml of 0.1 M Na_2CO_3 and 0.5 gm of twice recrystallized 2,4-dinitrobenzene sulfonate, Na salt (Eastman Organic Chemicals, Rochester) was added. After allowing the reaction to proceed for 9 days at 4^oC, the DNPI was precipitated at pH 5.5 by the addition of 6 N HCl. The yellow precipitate was dissolved in 1 M $NaHCO_3$ and chromatographed on a Sephadex G-25 column (100 ml), equilibrated with distilled water. The yellow fractions coming out at void volume were pooled, adjusted to pH 1.8 with 1 N HCl, and the protein precipitated with an equal volume of 5 M NaCl. This precipitation was repeated and the final DNPI precipitate dissolved in a small volume of 1 M $NaHCO_3$, filtered through a 0.45 μ Millipore membrance filter and stored at 4^oC. Before use, samples were dialysed against 0.01 M phosphate-buffered saline (PBS), pH 7.4 for at least 24 hours.

Preparation of ^{125}I-DNP-Insulin

The method used for external labelling of DNPI with ^{125}I was that described by Yagi (1971). To 0.25 ml of a DNPI solution (85 μg, 10^{-8} mole) in 0.2 M borate buffer, pH 8.0, 0.02 ml of ^{125}I (New England Nuclear, Mass., carrier free, 100 mc/ml) and 0.1 ml of KI (10^{-8} mole) were added. While the mixture was being stirred by a magnetic stirrer, 0.1 ml of chloramine T (2.5×10^{-7} mole; Eastman Organic Chemicals, Rochester) was added. After 5 minutes, the reaction was stopped by adding 0.1 ml of $NaHSO_3$ (5×10^{-7} mole). Starch KI paper was used to test whether enough $NaHSO_3$ had been added. The

mixture was applied to a small Sephadex G-25 column (12 x 100 mm), equilibrated with borate buffer. This column had been pretreated with 2 ml of a 2% solution of bovine serum albumin in order to saturate the protein binding sites of the gel. The excess BSA was washed out with 20 ml of borate buffer. Radioactive insulin was eluted with borate buffer and collected in 1 ml fractions. The radioactivity was estimated by placing each tube at a fixed distance from a Geiger-Müller detecter (Nuclear-Chicago). The radioactive peak fractions were pooled and dialyzed against 0.01 M PBS, pH 7.4 for 24 hours. After dialysis, the concentration of ^{125}I-DNPI was determined spectrophotometrically. ^{125}I-DNPI was stored in 0.5 ml portions at 0°C; some portions were mixed with 1% BSA in borate buffer.

The specific activity of ^{125}I-DNPI was estimated by counting the radioactivity in 1 ml of solution of known concentration using an Automatic Gamma Counter (Nuclear-Chicago).

Characterization of ε-DNP-Insulin

(A) Degree of Substitution by DNP

The degree of substitution of insulin by DNP was estimated by spectrophotometric measurement at two different wavelengths, i.e., 280 mμ and 360 mμ (Eisen et al., 1952). The ratio of extinction coefficients at 360 mμ to 280 mμ was taken as 2.89. The extinction coefficients for ε-DNP-lysine at 360 mμ and insulin at 280 mμ were taken as $E^{M}_{1cm} = 1.753 \times 10^{4}\ M^{-1}\ cm^{-1}$ (Little and Donahue, 1968) and $E^{1\%}_{1cm} = 10$ (Porter, 1953), respectively. The molecular weight of the insulin monomer was taken as 5750 (Sanger, 1955).

(B) Molecular Weight of ε-DNP-Insulin

(1) Gel Filtration Chromatography - The molecular weight of DNPI was determined according to the method of Andrews (1964). A Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, Calif.) column (2.5 x 60 cm) was equilibrated with barbital-NaCl buffer ($\Gamma/2 = 0.1$, pH 9.0). Dextran blue (Pharmacia, Uppsala, Sweden) or BGG (Bovine Gamma Globulin) (Nutritional Biochemicals Corp., Ohio) and DNP-lysine (Mann Research Laboratories, N.Y.) were used to determine the void volume (Vo) and the internal volume (V_i), respectively. The distribution coefficient (K_d) was obtained from the following relationship:

$$K_d = \frac{V_e - V_o}{V_i}$$

where V_e is the elution volume of the samples.

The Bio-Gel P-60 column was calibrated with lyophilized bovine ribonuclease A [(Type II-A, Sigma Chemical Co., St. Louis), which had been re-purified on a Bio-Rex 70 column according to the procedure of Hirs et al. (1956)], myoglobin (Whale muscle, Seravac Laboratories, Colnbrook, England) and ovalbumin (Turkey egg, 5 x crystallized, Pentex Inc., Ill.). The elution volume of each standard protein was determined and the K_d value calculated. The values of $(K_d)^{1/3}$ were then plotted against $(M.W.)^{1/2}$ following the method of Andrews (1964).

DNPI at two different concentrations was also run on a smaller Bio-Gel P-60 column (2.5 x 30 cm) equilibrated with 0.01 M PBS, pH 7.4. The concentration of eluted DNPI was monitored at 280 mμ and 360 mμ . The approximate molecular weight was determined from the elution volume and standard curves supplied by Bio-Rad Laboratories, Richmond, Calif. (1971).

(2) Thin-Layer Gel Chromatography - The molecular weight of DNPI was also estimated by thin-layer gel chromatography (TLG). These experiments were all done on a Pharmacia TLG-Apparatus (Figure 3) which can hold two 20 x 20 cm glass plates and the chamber of which can be adjusted to any angle from 10° to 40°.

Methods for swelling and spreading the gel strictly followed the recommendation of the manufacturer (Pharmacia Fine Chemicals, 1971). In a typical run, 6.5 gm of Sephadex G-100 (Superfine) was swollen in 100 ml of 0.01 M PBS, pH 7.4 on a boiling water bath for 3 hours, with occasional swirling of the beaker. The swollen gel was then spread on the glass plate with a TLG-spreader (Pharmacia) set at a thickness of 0.6 mm. Spreading was done by no more than two push-strokes. After placing the plate carefully into the chamber, the top, centre and bottom reservoirs were filled with 70, 70 and 25 mls of PBS, respectively. Paper bridges (Whatman 3 MM, 18 x 4.6 cm, fully saturated with PBS) were put in the top, centre and bottom reservoirs, with about 1.5 cm of the paper extending onto the gel plates. The TLG-chamber was set at a 10° angle and equilibrated overnight.

After equilibration, the TLG-chamber was placed on a horizontal level. The starting line was marked and about 5 - 10µ l of each protein (about 50 µg for reference proteins) sample was applied to the gel plate with the aid of Drummond "microcaps" micropipettes (10 µl), leaving a space of 2 cm between each sample. For application of DNPI, the same amount of 1% BSA was applied prior to DNPI. The reference proteins used in this experiment are listed in Table IV. After application, the TLG-chamber was again set at a 10° angle and the separation was then allowed to proceed for about 4 hours, by which time,

FIGURE 3 Thin-layer Gel Chromatography apparatus (Pharmacia, Uppsala, Sweden).

TABLE IV

STANDARD PROTEINS USED IN THIN-LAYER GEL CHROMATOGRAPHY

Protein	Mol. Wt.*	Source
RNase A	13,700	Type IIA, Sigma Chemical Co. Repurified by Bio Rex 70 resin and lyophilized.
Myoglobin	17,800	1 x crystallized, whale sperm, Seravac Laboratories.
α-Chymotrypsin	24,800	Type II, 3 x crystallized, Bovine pancreas, Sigma Chemical Co.
Pepsin	32,700	Worthington Biochemical Corp.
Ovalbumin	45,000	5 x crystallized, turkey egg, Pentex Inc.
BSA	65,000	Crystallized, Armour Pharmaceutical Co.
BGG	160,000	Fraction II, Nutritional Biochemicals Corp.

* Molecular weights were taken from Andrews (1965), Ackers (1964) and Putnam (1965).

the brown colored myoglobin (standard protein) had migrated to the midpoint (6 - 7 cm) of the gel plate.

At the completion of the run, the plates were removed. Proteins were detected and stained, essentially following the methods of Williamson and Allison (1967), with a minor change. Plates were put into an oven at 50 - 55°C for 10 minutes to remove most of the water, and were then dried under a stream of air from a table-fan for 1 - 2 hours. It was found that this method of drying prevented the surface from cracking, which usually occurred if Williamson and Allison's method was followed directly after removal of the plates from the TLG-chamber. The dried plates were immersed for 15 minutes in a saturated solution of Amido Black 10 B (Chroma-Gesellschaft, Stuttgart, W. Germany) in methanol: glacial acetic acid: water (50/10/40, v/v/v). Subsequent de-staining was performed in the dye solvent by 2 to 3 successive washes of 10-15 minutes each. The positions of the stained proteins were determined and the migration distance of the sample spot from the origin was measured. The "print" technique on Whatman 3 MM paper has also been tried. While the transfer of reference proteins to the paper was very successful, DNPI did not transfer, probably due to its low molecular weight and low concentration ($\simeq 10^{-4}$ M) (Radola, 1968).

Autoradiography

Since neither the dried plate method nor the filter paper "print" technique could identify DNPI on the TLG at concentrations of 10^{-6} - 10^{-7} M which were employed in the equilibrium and kinetic studies, TLG experiments were also performed with ^{125}I-DNPI. These were carried out essentially as described in the previous section.

^{125}I-DNPI was applied at a concentrations of 5×10^{-7} - 1×10^{-6} M. After the gel plates had been stained and dried, the migration distances of the reference proteins were measured. ^{125}I-DNPI did not stain at this concentration. An X-ray film (Kodak No Screen Medical X-ray Film, NS-2T, 8 x 10 inches) was placed on the gel plate, and fastened by Scotch tape. To shield the film from external radiation, the plate and film were then wrapped in three kinds of envelopes - first, a metal-coated envelope from the original film; second, a specially made black, thick paper envelope and finally an ordinary mail envelope. To make proper contact between the film and the plate, the two were sandwiched between two plywood boards, having inner linings of polyurethane foam sheets. Pressure was applied by screw nuts at the four corners.

After 2 days, the exposed film was developed by a two-step developer, Diafine (Acufine, Ill.), and fixed by ordinary film fixer.

(C) Isoelectric Focusing of ε-DNP-Insulin

In order to assess the charge homogeneity of DNPI, it was analysed by isoelectric focusing. This experiment was kindly performed by Dr. E. R. Centeno of the Department of Immunology, using an LKB 8101 electrofocusing column. A stabilizing gradient of 0 to 46% sucrose (Schwarz/Mann, Orangeburg, N.Y.) was used. Separation was performed in a pH gradient with the range of pH 3 to pH 10 employing a carrier ampholyte supplied by LKB, Stockholm, Sweden. Prior to electrofocusing, DNPI at a concentration of 0.5 mg/5 ml, was dialyzed against 0.1 M glycine, pH 6.0. The pH of the eluted fractions were measured with a Beckman Research pH Meter and the optical density was monitored using an LKB Uvicord II absorptiometer at 280 mμ as well as a Zeiss PM QII spectrophotometer at 280 mμ and 360 mμ .

Preparation of Antigen

BGG-DNP was prepared by coupling 2,4-dinitrobenzene sulfonate (Eastman Organic Chemicals, Rochester) to bovine gamma globulin (Nutritional Biochemicals Corp., Ohio) according to Eisen et al. (1952). The substituted BGG-DNP was separated from the uncoupled hapten on a column of Sephadex G-25 and the BGG-DNP was then exhaustively dialyzed against 0.01M PBS, pH 7.4. The degree of coupling was determined spectrophotometrically from the optical density of the BGG-DNP solution in 0.01M NaOH at two different wavelengths: 280 m μ and 360 m μ (Eisen et al., 1952).

Immunization Procedure

New Zealand white rabbits were immunized with 10 mg of BGG-DNP in complete Freund's adjuvant (Difco Laboratories, Detroit). After 2 weeks, the rabbits received weekly booster injections of 10 mg 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 2 months, a booster injection of 10 mg antigen was given to the animals; they were again bled after 7 days. The antisera were pooled and Seitz-filtered into sterile vials for storage in the frozen state.

Preparation of Antibodies

Anti-DNP antibodies* were purified on the specific immunoabsorbent, DNP-HSA-bromoacetyl cellulose which was prepared according to the method of Robbins et al. (1967). Antibodies were eluted with 0.01M 2,4-dinitrophenol (DNP-OH) (Merck, Darmstadt, Germany) adjusted to pH 8.0. The DNP-OH was then removed by passing the eluate

* This purified antibody preparation was kindly provided by Dr. K. Kelly.

through an anion exchange column Dowex 1 x 8 (Cl^-, 200-400 mesh) equilibrated with phosphate buffer, pH 7.4.

Preparation of Fab' Fragments

$F(ab')_2$ fragments from purified antibody were prepared according to Nisonoff et al. (1959). To 100 mg of antibody in 6 ml of acetate buffer, pH 4.5, 3.5 mg of pepsin (Worthington Biochemical Corp., N.J.) were added and hydrolysis was allowed to proceed at 37°C for 18 hours. The reaction was stopped by adjusting the mixture to pH 8.0; it was then applied to a Bio-Gel P-200 column (2.5 x 100 cm) equilibrated with Tris-HCl buffer ($\Gamma/2 = 0.1$, pH 8.0). The purity of the $F(ab')_2$ preparation was checked by ultracentrifugation in a Beckman Model E analytical centrifuge. Finally, the $F(ab')_2$ preparation was dialysed against distilled water for 48 hours and lyophilized.

Reduction of $F(ab')_2$ to Fab' fragments was done according to Nisonoff (1964). A purified $F(ab')_2$ preparation at a concentration of 1% (w/v) was dialysed against 0.1M acetate buffer, pH 5.0, at 4°C for 48 hours. The reduction was accomplished by addition of 0.1M mercaptoethylamine hydrochloride to give a final concentration of 0.015 M. This mixture, contained in a stoppered vial, was flushed with pure N_2 gas. The vial was then closed tightly and incubated in a constant water bath at 37°C for 90 minutes. The reaction was stopped by adding 0.2 M iodoacetate (Na salt) to a final concentration of 0.02 M. The mixture was finally dialysed against 0.01 M PBS, pH 7.4 in 4°C for 48 hours and stored in a sterile vial in 4°C. The purity of this Fab' preparation was checked by ultracentrifugation in a Beckman Model E analytical centrifuge.

Equilibrium Study - Fluorescence Titrations

The specific binding of DNPI by the purified antibody and its Fab' fragments was measured by the fluorescence quenching technique according to Velick et al. (1960), using an Aminco-Bowman spectrophotofluorometer. An excitation wavelength of 290 mμ was used, while fluorescence was measured at 350 mμ . All glassware used for handling the solutions in these experiments was cleaned in concentrated nitric acid. The 0.01 M PBS, pH 7.4, was prepared with double distilled water. The antibody and DNPI solutions were dialyzed against the PBS at 4°C for at least overnight before being used. They were then passed through a Millipore filter (0.45 μ) and degassed under vacuum just prior to the titration experiments. A volume of 2 ml antibody solution was added to a quartz cuvette maintained at a constant temperature of 25°C. The initial fluorescence value was recorded after temperature equilibration had been achieved (10 minutes) and DNPI was then added by means of an "Agla" micrometer syringe in increments of 0.002 to 0.004 ml to a total of 0.2 ml.

After each addition of DNPI, the solution was gently stirred by means of a platinum coil (usually 10 strokes) and the fluorescence value was recorded after a fixed interval of 30 seconds, for thermal equilibration. All titrations were carried out in duplicate or triplicate.

For determination of the maximum quenching of fluorescence, Q_{max}, the antibody solution (40-50 μ g/ml) was titrated with a high concentration of DNPI (5×10^{-4} M) to a final concentration approximately 50 fold in excess over antibody site concentration, without

introducing a large dilution. For the determination of binding constants, the antibody solution (100 μg/ml) was titrated with a lower concentration of DNPI (1×10^{-4} M).

Non-specific collision quenching and attenuation of incident and emergent radiation by DNPI were corrected from fluorescence values of control titrations of nonspecific rabbit IgG with DNPI. A blank value was obtained from the fluorescence of solvent alone (usually less than 5% of the initial antibody fluorescence). This blank fluorescence value was substracted from the emitted fluorescence which was then corrected for dilution, and normalized for 100 per cent initial fluorescence intensity. Correction of DNPI attenuation was made according to the method of Kelly (1970). The logarithm of the fluorescence intensity was plotted against DNPI concentration, and the slope in the DNPI excess region extrapolated to zero concentration of DNPI. The Q_{max} value, considered to represent the maximum quenching when 100 per cent of the antibody combining sites were occupied by DNPI, was the antilog value of the intercept.

To obtain the actual binding data, the corrected relative fluorescence intensity (RFI) was plotted as a function of DNPI concentration. An extrapolation of the initial slope of this curve to an RFI value which corresponded to Q_{max}, yielded the antibody site concentration (Ab_T) in the sample at equivalence. The fraction of antibody sites occupied, r, at a particular concentration of DNPI, (H_T), was obtained from the relationship:

$$r = \frac{Q}{Q_{max}} \qquad (13)$$

where Q is the observed quenching produced by bound DNPI. The concentration of free DNPI (c), was determined from the difference between the concentration of total DNPI added (H_T) and bound DNPI (rAb_T):

$$c = (H_T) - r(Ab_T) \qquad (14)$$

The average intrinsic association constant, K_o^a, and the heterogeneity index (α) were determined from a plot of log ($\frac{r}{1 - r}$) against log (c) according to Sips equation in its logarithmic form as suggested by Karush (1962):

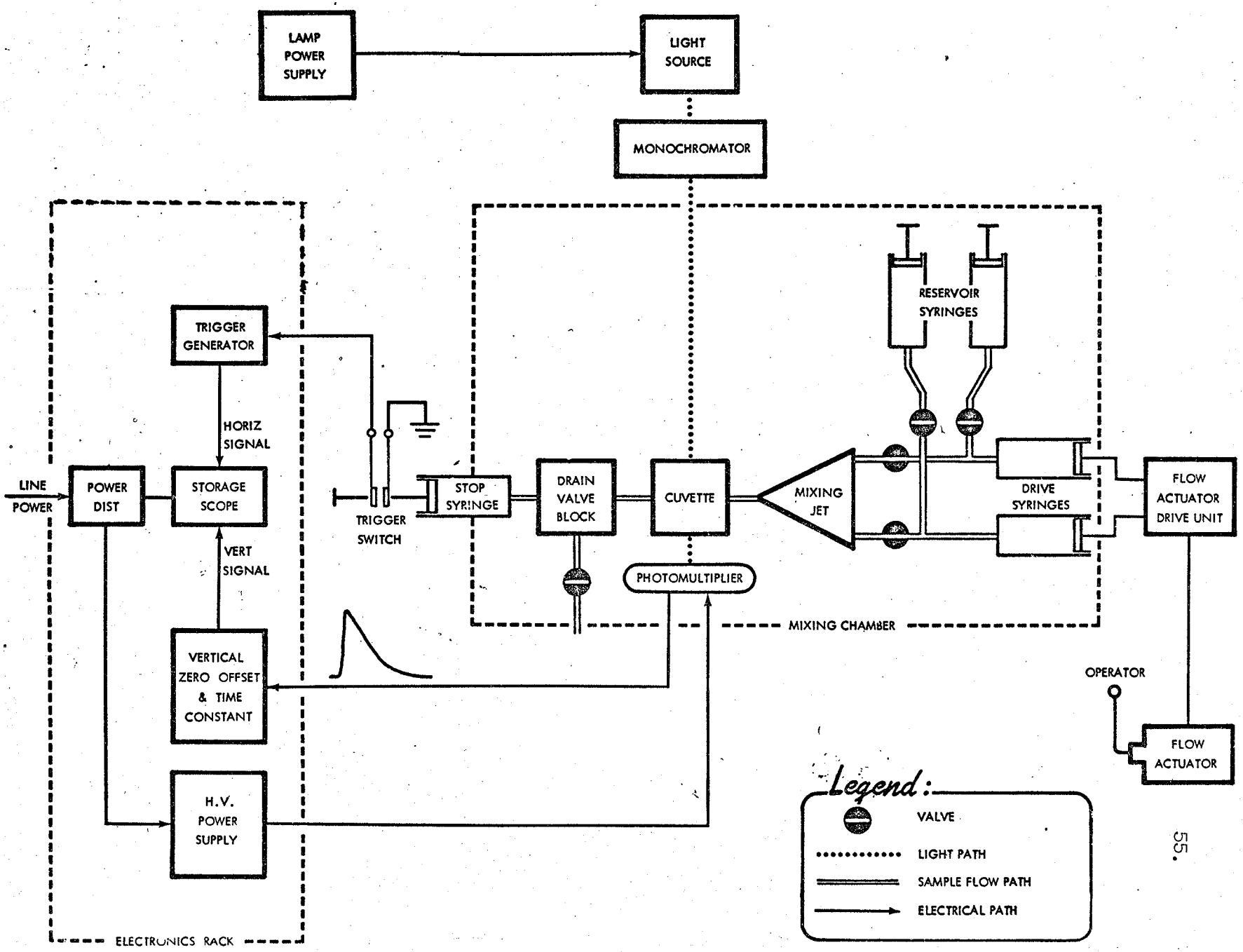
$$\log (r/1 - r) = \alpha \log c + \alpha \log K_o^a \qquad (15)$$

Kinetic Study - Stopped-Flow

Rate measurements of the quenching offluorescence of antibody by the univalent antigen DNPI were carried out at 25°C in 0.01 M PBS, pH 7.4 with the aid of a Durrum-Gibson stopped-flow spectrophotometer (Durrum Instrument Corp., Palo Alto, Calif.). A functional block diagram of the stopped-flow system is given in Figure 4.

The reaction components were stored in two large reservoir syringes (20 ml) which in turn were connected through valves to the 2 ml drive syringes. These syringes were connected with the Kel-F mixing jet outlet in a valve block. The quartz reaction cuvette had dimensions of 2 x 20 mm. The outlet from the cuvette was connected through a drain valve block, made of stainless steel, to the "stop" syringe. To the plunger of this syringe, an adjustable external "stop" was attached. This "stop" not only halted the flow after a certain predetermined volume had entered the syringe, but also actuated the trigger switch for the oscilloscope. A valve-

FIGURE 4 Schematic diagram of the stopped-flow apparatus.


LAMP POWER SUPPLY
LIGHT SOURCE
MONOCHROMATOR
RESERVOIR SYRINGES
TRIGGER GENERATOR
HORIZ SIGNAL
LINE POWER
POWER DIST
STORAGE SCOPE
TRIGGER SWITCH
STOP SYRINGE
DRAIN VALVE BLOCK
CUVETTE
MIXING JET
DRIVE SYRINGES
FLOW ACTUATOR DRIVE UNIT
VERT SIGNAL
PHOTOMULTIPLIER
MIXING CHAMBER
VERTICAL ZERO OFFSET & TIME CONSTANT
OPERATOR
FLOW ACTUATOR
H.V. POWER SUPPLY
ELECTRONICS RACK
Legend:
VALVE
LIGHT PATH
SAMPLE FLOW PATH
ELECTRICAL PATH

controlled drain port was also connected to the drain valve block for removal of the reacted solution.

The light source was 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, and a stable current was maintained at 6 - 8 amps by a rheostat. In fluorescence measurements, the light, after passing through a prism monochromator, entered the reaction curvette at right angles to the exit path. A filter (# 2, 1% transmittance at 295 m μ, 70% transmittance at 325 m μ) was also installed on the photomultiplier housing light entrance tube, to prevent residual excitation radiation (λ <325 m μ) from reaching the photomultiplier. This 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 2A 63, and displayed on a Tetronix type RM 564 storage oscilloscope.

The drive syringes, valve block and cuvette were maintained at constant temperature in a closed circuit, open reservoir, circulating bath controlled by a Lauda K 2/R thermostat.

In an actual run, an excitation wavelength of 280 m μ was used, the flow system was flushed with nitric acid and immediately rinsed with double-distilled water and finally with PBS. The solutions of the reactants were filtered through Millipore filters (0.45 μ), degassed and transferred to their respective reservoir syringes.

After the drive syringes had been filled with the reactants, the solution was pushed back and forth between its drive and reservoir syringes to dislodge any trapped air bubbles. After allowing 10 minutes for the solutions to reach constant temperature (25°C), the flow actuator handle was struck sharply forcing equal volumes (0.3 ml) of the reactants to pass through the flow system to the stop syringe. The antibody fluorescence level at its equilibrial state with DNPI was also obtained as a control, about 2 minutes after the mixing of reactants. Once the run was good as judged by the oscilloscope display, the tracing was recorded on Polaroid film (Polaroid, land pack film type 107, $3\frac{1}{4}$ x $4\frac{1}{4}$ inches).

Each reaction trace was transposed onto tracing paper and smoothed by hand. The data were treated according to Day et al. (1963). On the assumption that all antibody sites are equivalent and independent, the general rate expression for a reaction of the type shown in equation (11) can be expressed by the relationship:

$$\log \left(\frac{1 - \phi x}{1 - x} \right) = k_{12} \frac{\sqrt{-Q}}{2.303} t \qquad (16)$$

where

$$\phi = \frac{a_o + b_o + K_o^d - \sqrt{-Q}}{a_o + b_o + K_o^d + \sqrt{-Q}}$$

$$\sqrt{-Q} = [(b_o - a_o + K_o^d)^2 + 4 a_o K_o^d]^{\frac{1}{2}} \; ; \; b_o \geq a_o$$

a_o = initial antibody binding site concentration

b_o = initial DNPI concentration

K_o^d = intrinsic dissociation constant $= \frac{1}{K_o^a}$

x = fractional extent of reaction toward equilibrium at time t; $x = 0$ at $t = 0$; $x = 1$ at $t = \infty$

The rate constant, k_{12}, was then obtained from the slope of a plot of log $[(1 - \phi x)/(1 - x)]$ versus (mt), where $m = \sqrt{-Q} / 2.303$. In such a plot, data obtained for various initial concentrations of antigen and antibody could be combined.

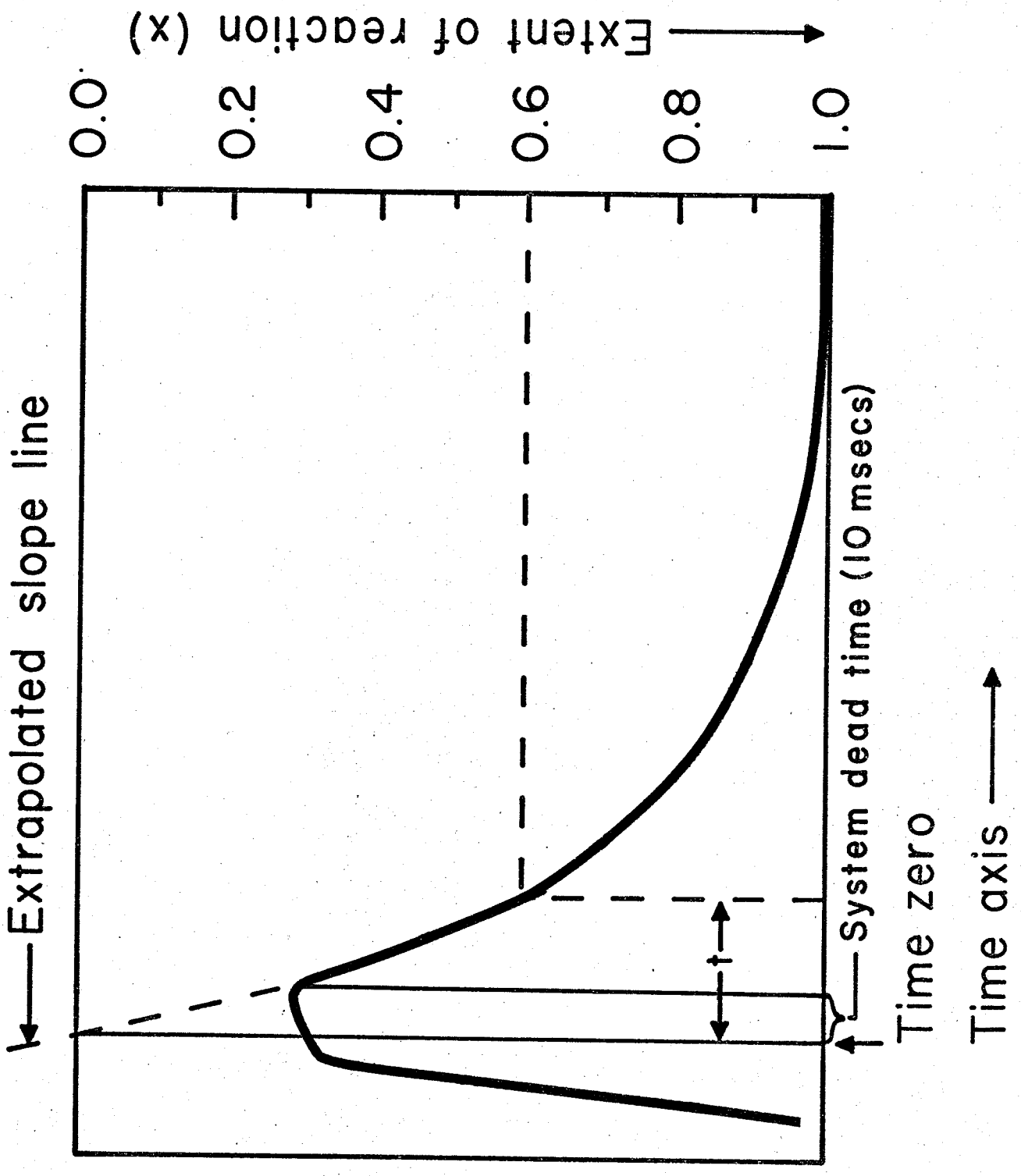
The reverse rate constant, k_{21}, was determined from the relationship:

$$k_{21} = k_{12}/K_o^a$$

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 the method of Day et al. (1963), 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-antigen mixture at zero time of reaction, since the attenuation by antigen under experimental conditions was negligible. The initial slope of the reaction-produced fluorescence drop-off would then be extrapolated to the initial fluorescence level (see Figure 5). The time, on the horizontal time base axis, from this initial level to the beginning of the observed fluorescence drop-off (the system dead time) would correspond to the average age of the mixed solution when it came to rest in the reaction cuvette.

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 reaction. Therefore, a system consisting of bovine serum albumin (BSA) and the

FIGURE 5 Analysis of a reaction waveform.


Extent of reaction (x)
0.0
0.2
0.4
0.6
0.8
1.0
Extrapolated slope line
System dead time (10 msecs)
Time zero
Time axis

dye, 1-hydroxy-4-(2,4-dinitrophenylazo)-2,5-naphthalene disulfonate (1N-2,5S-4DNP), in phosphate buffer (pH 6.0, $\Gamma/2 = 0.1$) was used to approximate the dead time, under experimental conditions. The absorbance of the dye in buffer at 470 mμ was reduced markedly in the presence of BSA. These experiments could be performed with the more stable tungsten-iodide lamp, under flow conditions identical to those used in fluorescence runs. Instrument dead time from this system could then be used to determine the initial fluorescence level at zero time of reaction of antibody with DNPI.

RESULTS AND DISCUSSION

Degree of DNP Substitution in ε-DNP-Insulin

When DNPI was analysed by spectrophotometric measurements at two wavelengths (280 mμ and 360 mμ, respectively) for the number of 2,4-dinitrophenyl groups incorporated, it was found that there were about 0.5 groups DNP per mole (mol. wt. 5750) of insulin*. This degree of substitution agrees closely with the data of Little & Counts (1969) who showed by amino acid analysis and thin layer chromatography, that approximately 60% of insulin was unsubstituted, and only lysine 29 was substituted in each preparation.

Estimation of the Molecular Weight of ε-DNP-Insulin

(A) Gel Filtration Chromatography

To determine the molecular weight of DNPI, a standard curve was constructed from the data shown in Table V, as described by

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* The detailed calculationsare shown in Appendix A.

TABLE V

ELUTION VOLUME, DISTRIBUTION COEFFICIENT AND MOLECULAR WEIGHT OF EXPERIMENTAL PROTEINS

Protein	Ve	$K_d = \frac{Ve - Vo^{*}}{Vi^{**}}$	$K_d^{1/3}$	$(M.W.)^{\frac{1}{2}}$
Ovalbumin	113.17	0.039	0.337	202.48
Myoglobin	136.25	0.114	0.484	133.41
RNase A (monomer)	128.20	0.088	0.443	159.37
RNase A (dimer)	160.43	0.202	0.586	112.69
DNPI	174.93	0.239	0.619	100.00

* Vo was determined by BGG

** Vi was determined by ε-DNP-lysine

Andrews (1964) using several proteins of known molecular weight. This method makes use of the theoretical treatment of gel filtration due to Porath (1963) who derived the following relationship between the distribution coefficient K_d and molecular weight:

$$K_d^{1/3} = k \left[1 - k' \frac{M^{\frac{1}{2}}}{(S_r - w)^{1/3}} \right]$$

where

k and k' = proportionality constants

S_r = solvent regain of the gel

w = the part of S_r from which all solute molecule are excluded

The standard plot derived in this way is shown in Figure 6. From K_d value of DNPI it could be concluded that at a concentration of 1.8×10^{-4}M*, this molecule had a molecular weight of 10,000 and thus, existed as a dimer.

Using a smaller column (2.5 x 30 cm) the concentration dependence of the molecular weight of DNPI was estimated. Two concentrations of DNPI (1.7×10^{-4} M and 2.8×10^{-4} M) were applied. Theelution profiles are shown in Figure 7. From the elution volumes it was estimated that DNPI existed as a tetramer (Molecular weight = 24,000) and a dimer (Molecular weight = 12,000). As can be seen, the ratio of the two components exhibited a strong concentration dependence, the tetramer being predominant at higher protein

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* Estimated in terms of molecular weight of 12,000.

FIGURE 6 Determination of the molecular weight of DNPI on a Bio-Gel P-60 column (2.5 x 30 cm) in 0.01M PBS, pH 7.4. K_d = distribution coefficient, M.W. = molecular weight.

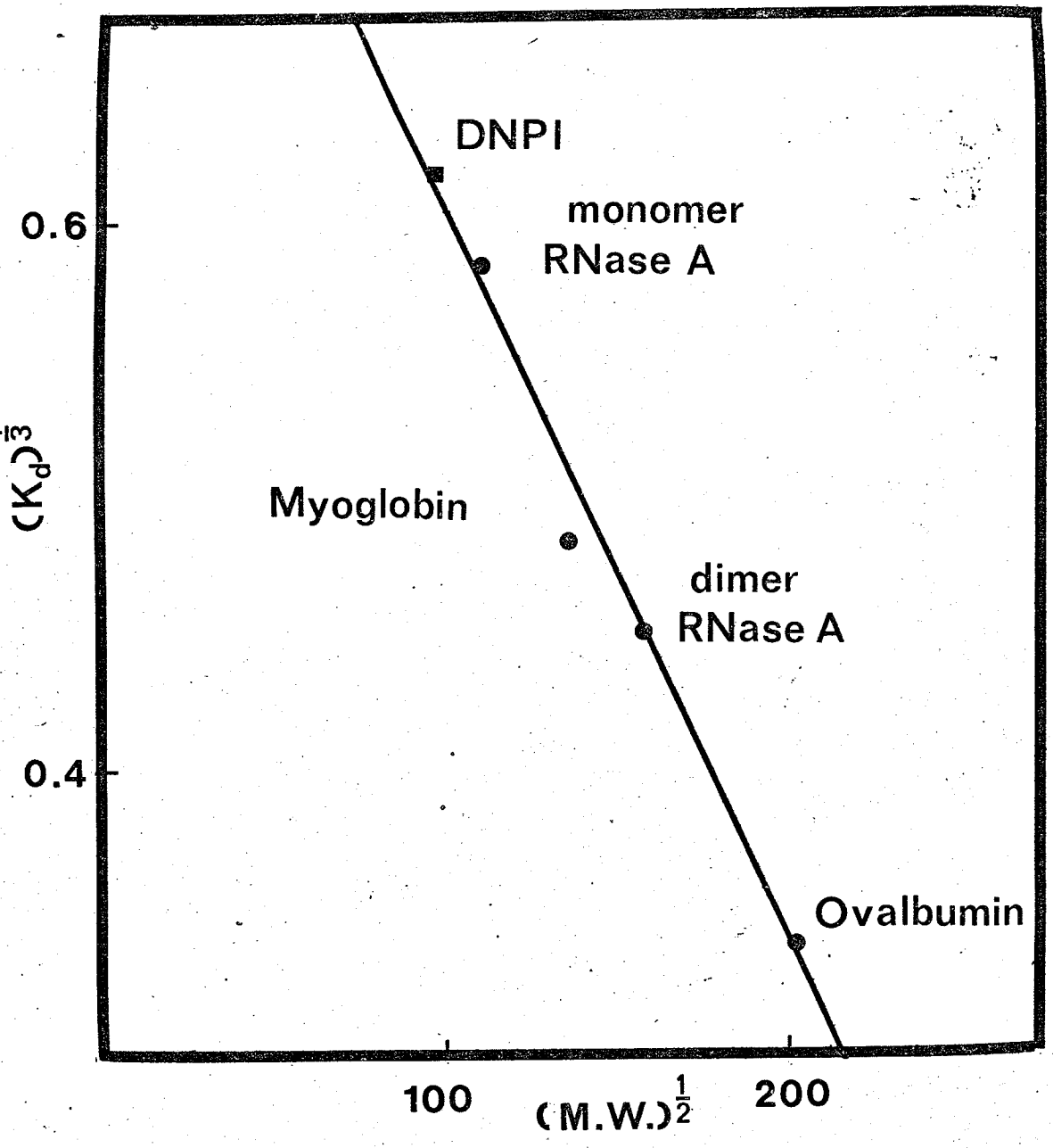
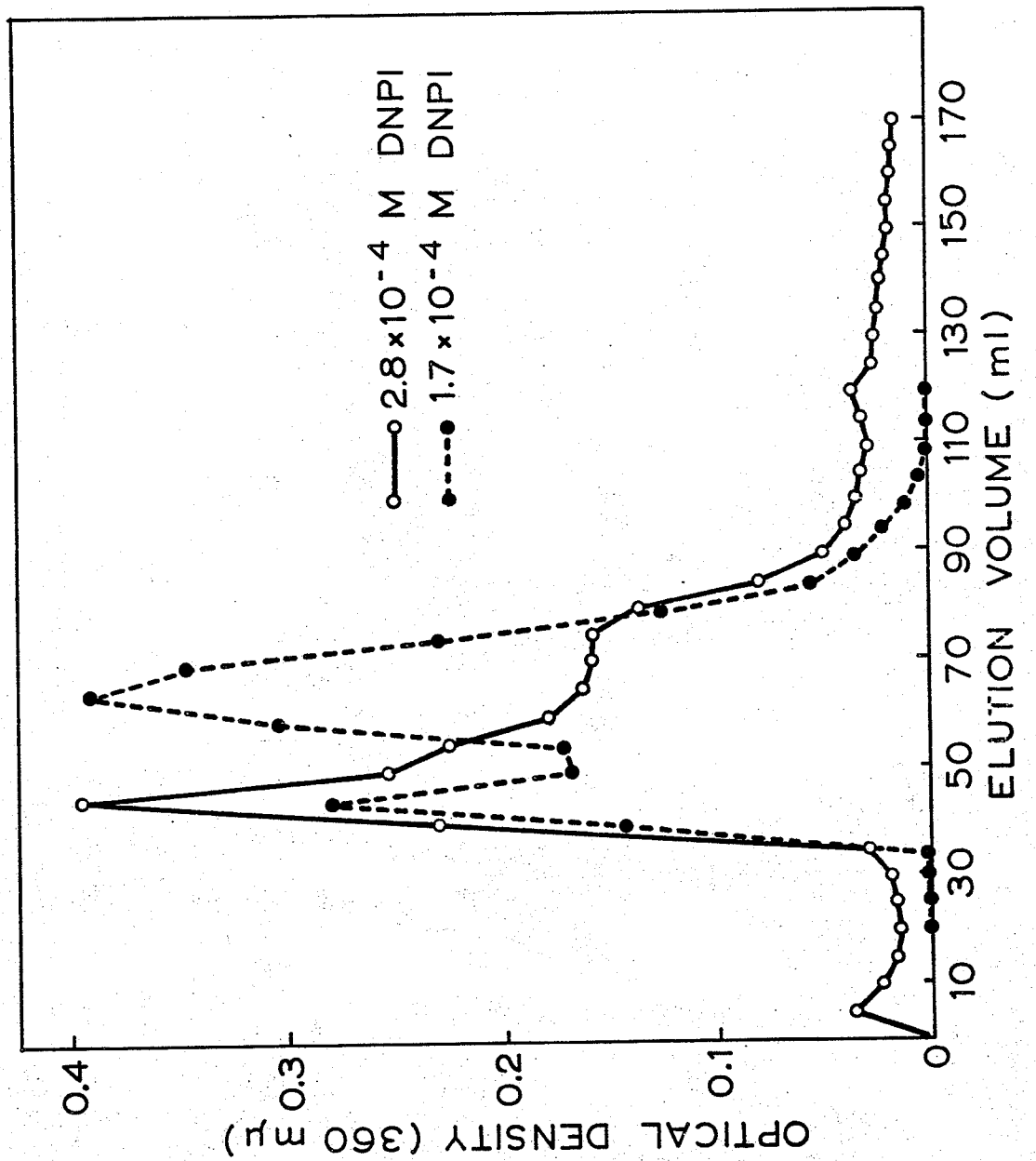

DNPI
monomer
RNase A
Myoglobin
dimer
RNase A
Ovalbumin
0.6
0.4
$(K_d)^{-\frac{1}{3}}$
100
$(M.W.)^{\frac{1}{2}}$
200

FIGURE 7 Elution profile of DNPI on a Bio-Gel P-60 column (2.5 x 60 cm) in barbital-NaCl buffer (pH 9.0, $\Gamma/2 = 0.1$), showing the concentration dependence of molecular weight of DNPI.


2.8×10⁻⁴ M DNPI
1.7×10⁻⁴ M DNPI
OPTICAL DENSITY (360 mμ)
0.4
0.3
0.2
0.1
0
10
30
50
70
90
110
130
150
170
ELUTION VOLUME (ml)

concentrations. A similar observation was made by Fredericq(1956) in the case of native insulin.

(B) Thin Layer Gel Chromatography

Since most equilibrium and kinetic experiments described below were performed at DNPI concentration of 10^{-6} - 10^{-7} M, it was of interest to determine the state of aggregation of DNPI at these low concentrations. The gel filtration experiments mentioned above could not be performed at concentrations much below 10^{-4}M. Therefore, it was decided to use the thin layer gel chromatography developed by Radola (1968). Unfortunately, when Amido Black was used to stain the protein spots on the thin layer plate, DNPI concentrations below 10^{-4} M could not be detected. Thus experiments were performed at 1 x 10^{-4} and 2 x 10^{-4} M. Figure 8 shows an actual run on a 20 x 20 cm plate. It should be pointed out that BSA was applied on the same spot prior to DNPI, in order to saturate the protein binding sites of the gel.

For the determination of molecular weights, the distance from the starting line to the middle of each zone for a particular protein was designated as d_p. Since the migration distance of myoglobin (d_M) was chosen as a standard, the ratio of d_p to d_M was defined as R_M:

$$R_M = \frac{d_p}{d_M}$$

R_M values for the reference proteins and DNPI with their molecular weights are shown in Table VI. A plot of $1/R_M$ as function of log molecular weight displayed a good linear relationship (Figure 9).

FIGURE 8 Thin-layer gel chromatogram of DNPI and several standard proteins on Sephadex G-100 (Superfine) in 0.01M PBS, pH 7.4. The arrow indicates the point of application of the samples.

1. Pepsin
2. Chymotrypsin
3. BSA
4. BSA (top), DNPI (1×10^{-4}M, bottom)
5. BSA (top), DNPI (1×10^{-4}M, bottom)
6. BSA (top), DNPI (2×10^{-4}M, bottom)
7. Myoglobin
8. Ovalbumin
9. RNase A

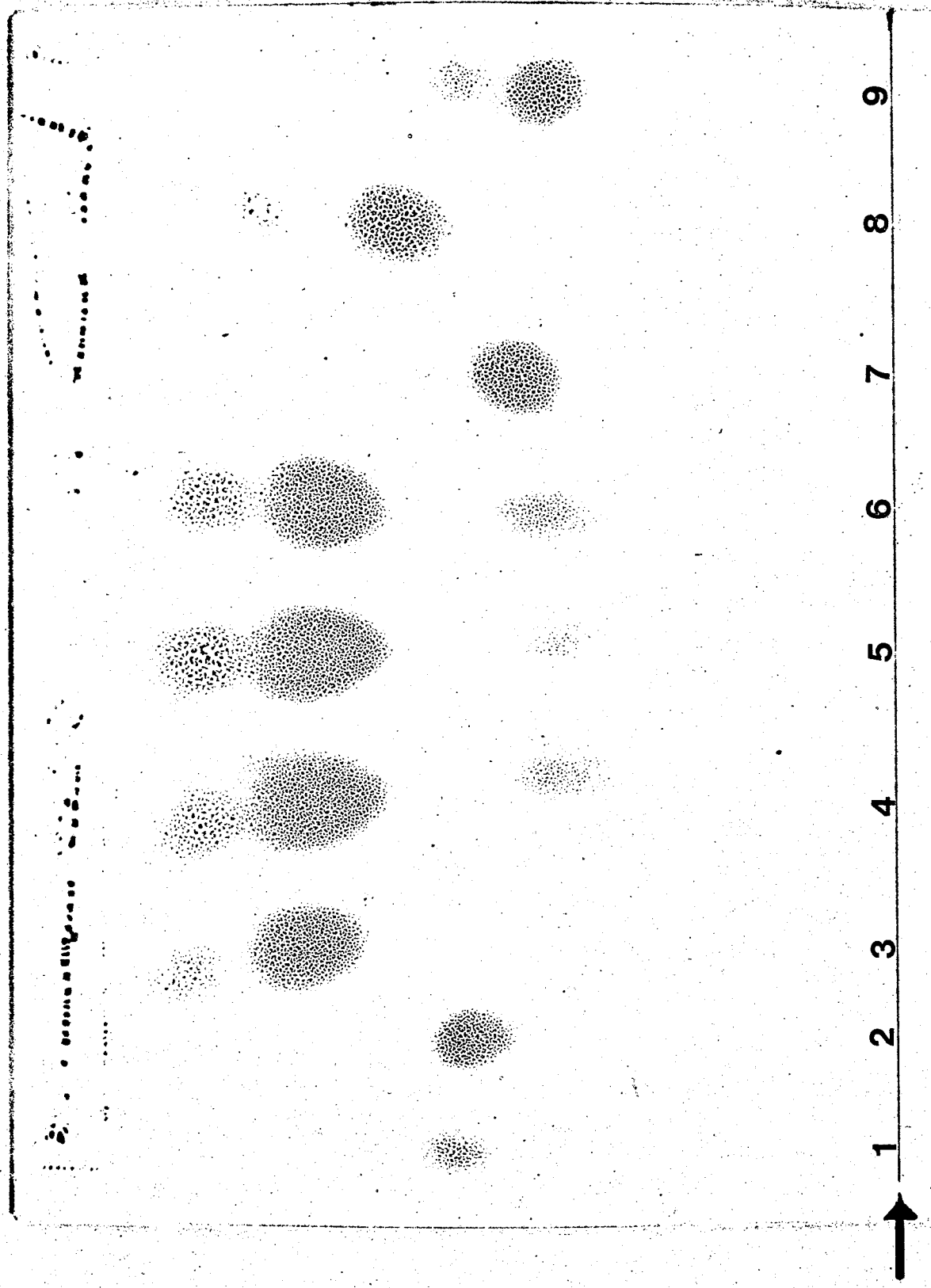

1
2
3
4
5
6
7
8
9

FIGURE 9 Determination of the molecular weight of DNPI by TLG on Sephadex G-100 (Superfine) in 0.01M PBS, pH 7.4.

R_M = migration distance relative to myoglobin.

M.W. = molecular weight.

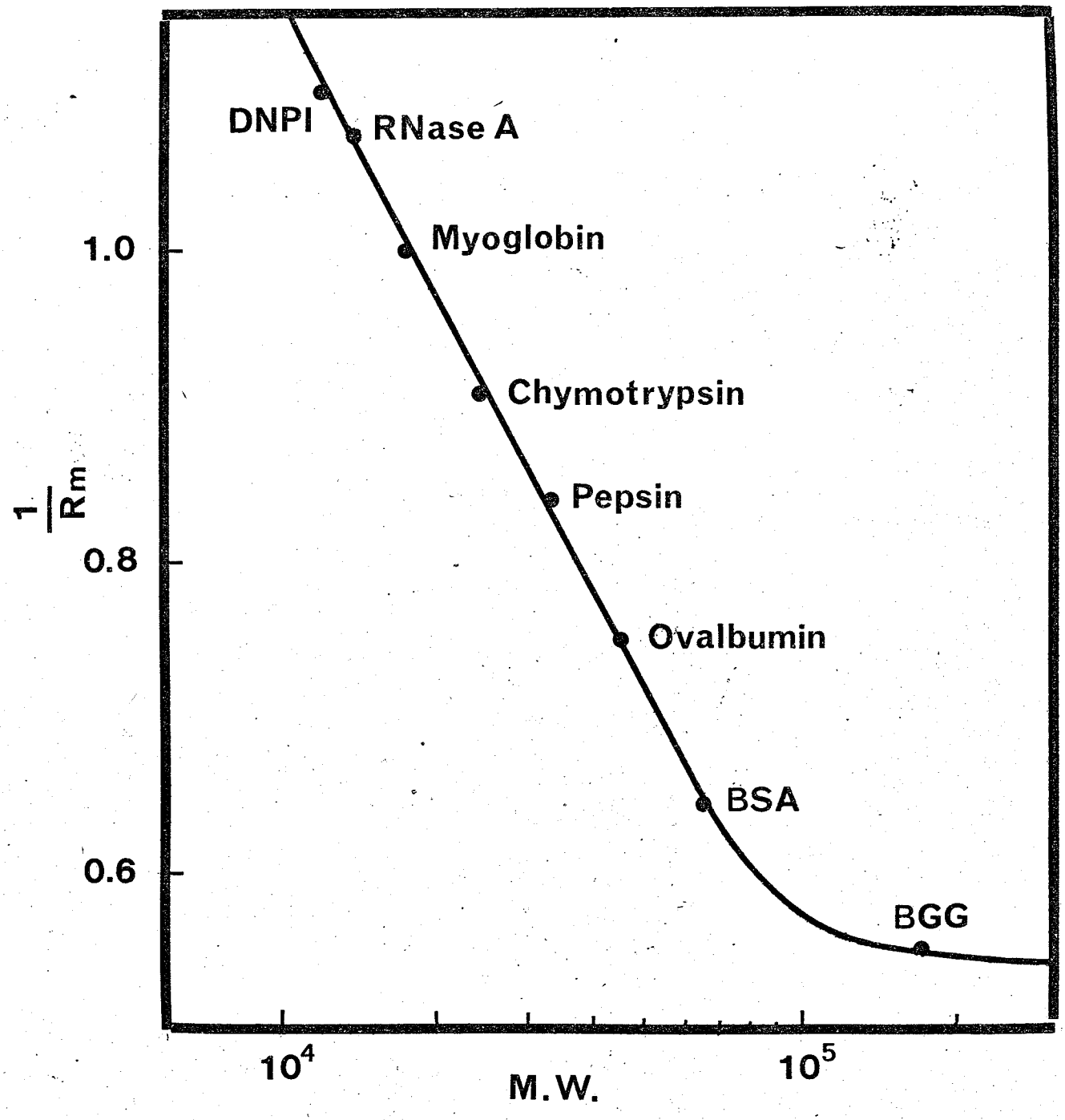

DNPI
RNase A
Myoglobin
Chymotrypsin
Pepsin
Ovalbumin
BSA
BGG
1.0
0.8
0.6
1/Rm
10^4
10^5
M.W.

TABLE VI

R_M VALUES AND MOLECULAR WEIGHTS OF SEVERAL PROTEINS ON SEPHADEX G-100 (SUPERFINE) BY TLG

Protein	Mol. Wt.	dp	R_M	$\frac{1}{R_M}$
RNase A	13,700	5.79	0.92	1.075
Myoglobin	17,800	6.28	1.00	1.000
Chymotrypsin	24,800	6.90	1.10	0.904
Pepsin	32,700	7.42	1.18	0.842
Ovalbumin	45,000	8.36	1.34	0.745
BSA	65,000	9.92	1.58	0.645
BGG	160,000	11.36	1.82	0.550
DNP-Insulin	12,000(dimer)	5.71	0.91	1.106

The molecular weight of DNPI, determined from this calibration line, gave a value of about 12,000, confirming the results obtained by column chromatography.

In order to detect lower DNPI concentrations on the thin layer plates, it was decided to use autoradiographic methods of detection. DNPI was radioactively labelled with ^{125}I by the chloramine-T method. Figure 10 shows the elution profile of ^{125}I-DNP-Insulin on a Sephadex G-25 column (1 x 14 cm) equilibrated and eluted with borate buffer, pH 8.0. An efficiency of iodination of 64% was calculated from this profile by dividing the radioactivity in the protein peak by the sum of radioactivity in protein and iodide peaks. The specific activity of ^{125}I-DNPI was found to be about 1 μC/μg of DNPI.

TLG on Sephadex G-100 (Superfine) was done as described under "Methods". ^{125}I-DNPI was applied in concentrations from 5.0×10^{-7} to 1.0×10^{-6} M after application a 1% solution of BSA. From a typical film (Figure 11), it was observed that the ^{125}I-DNPI did not move from the point of application, indicating that at these concentrations, most of the ^{125}I-DNPI was bound by either the Sephadex or by the glass.

Isoelectric Focusing of DNPI

The main reason for performing isoelectric focusing was to establish the number of charged species present in the DNPI preparation. Experiments designed to establish the degree of aggregation of DNPI (see above) had indicated that the maximum possible

FIGURE 10 Elution profile of ^{125}I-DNP-Insulin on a Sephadex G-25 column (12 x 100 cm) in borate buffer (0.2M, pH 8.0)

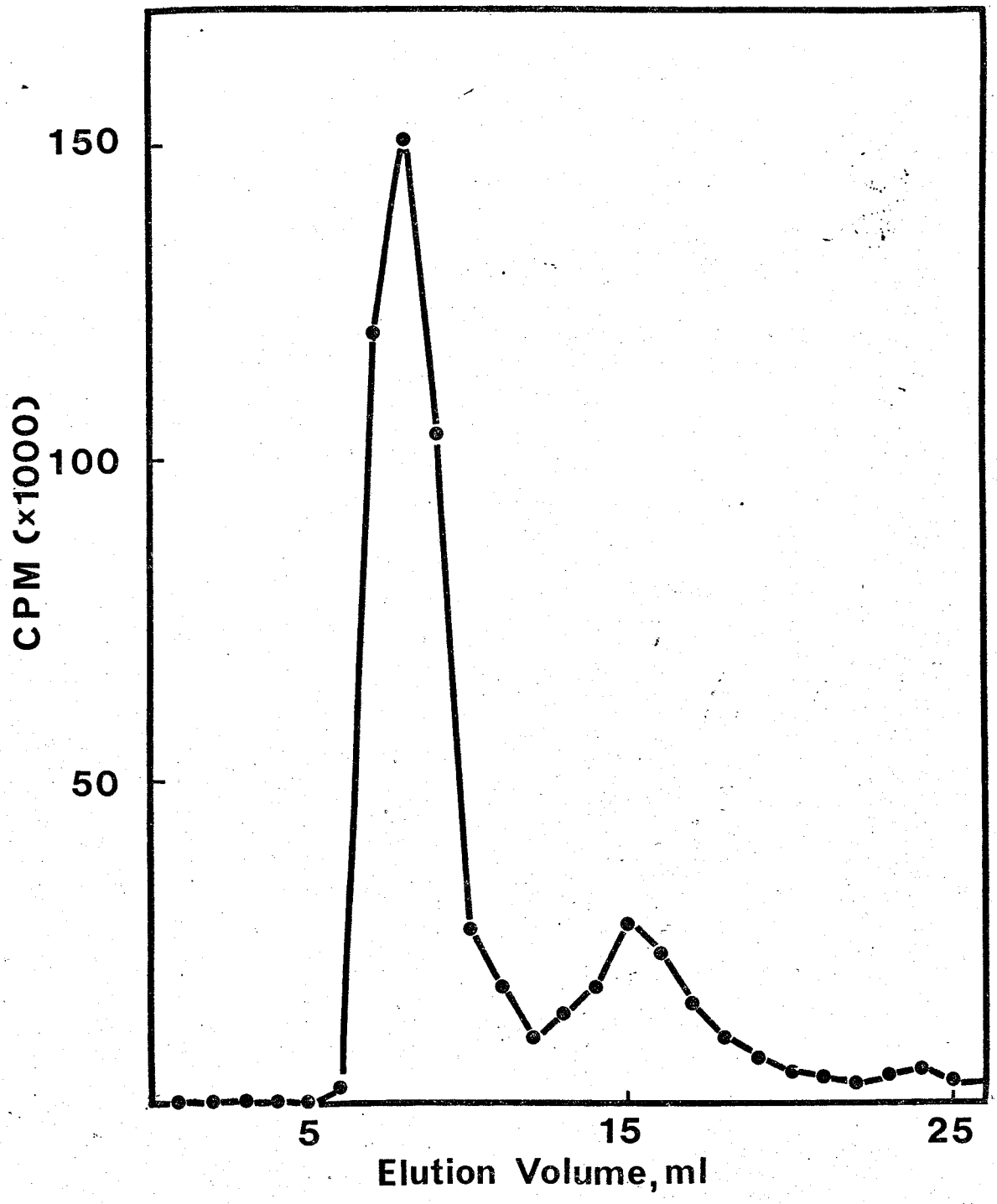

150
100
50
CPM (x1000)
5
15
25
Elution Volume, ml

FIGURE 11 Autoradiography of ^{125}I-DNP-Insulin on a Sephadex G-100 TLG plate. Arrow indicates migration direction. Concentrations of ^{125}I-DNP-Insulin (from left to right): 1×10^{-6}M, 1×10^{-6}M, 5×10^{-7}M.

molecular weight of DNPI at concentrations below 1 x 10^{-4} M was 12,000. Moreover, since it was calculated that one DNP group had coupled per insulin dimer, the possibility existed that the insulin preparation used for binding and kinetic experiments was composed of the following dimeric species: I-I, I-DNPI and DNPI-DNPI. Of this, the dimer withtwo DNP groups, when reacted with divalent antibody, could lead to the formation of larger antigen-antibody aggregates. Since the DNP had coupled to the only lysyl residue of the B chain, it was hoped that isoelectric focusing could yield information as to the presence of three species, which should have different isoelectric points, and which should also be distinguishable on the basis of the ratios of optical densities at 360 m μ to 280 mμ . After the DNPI preparation had been dialysed versus 0.1 M glycine, pH 6.0, and the ampholine-sucrose mixture added prior to electrofocusing, a large portion (≃ 90%) of the DNPI precipitated. The precipitate was centrifuged out and the supernatant was subjected to electrofocusing as described under "Methods". The elution profile of the electrofocused material is shown in Figure 12. A rather heterogeneous pattern was obtained. The peak at pH 5.5, the pI value for native insulin, was surprisingly small and, in addition exhibited some absorption at 360 mμ , making it rather difficult to assign this peak to unsubstituted insulin. Identification of the other peaks was also complicated by the fact that they all absorbed at 360 mμ, even though this absorption was relatively lower than that of the original DNPI preparation. Since the material, actually electrofocused, did comprise only about 10% of the starting sample, the

FIGURE 12 Isoelectric focusing elution profile of DNPI, pH 3-10. Optical density measured at 280 mμ (——) and 360 mμ (·····).

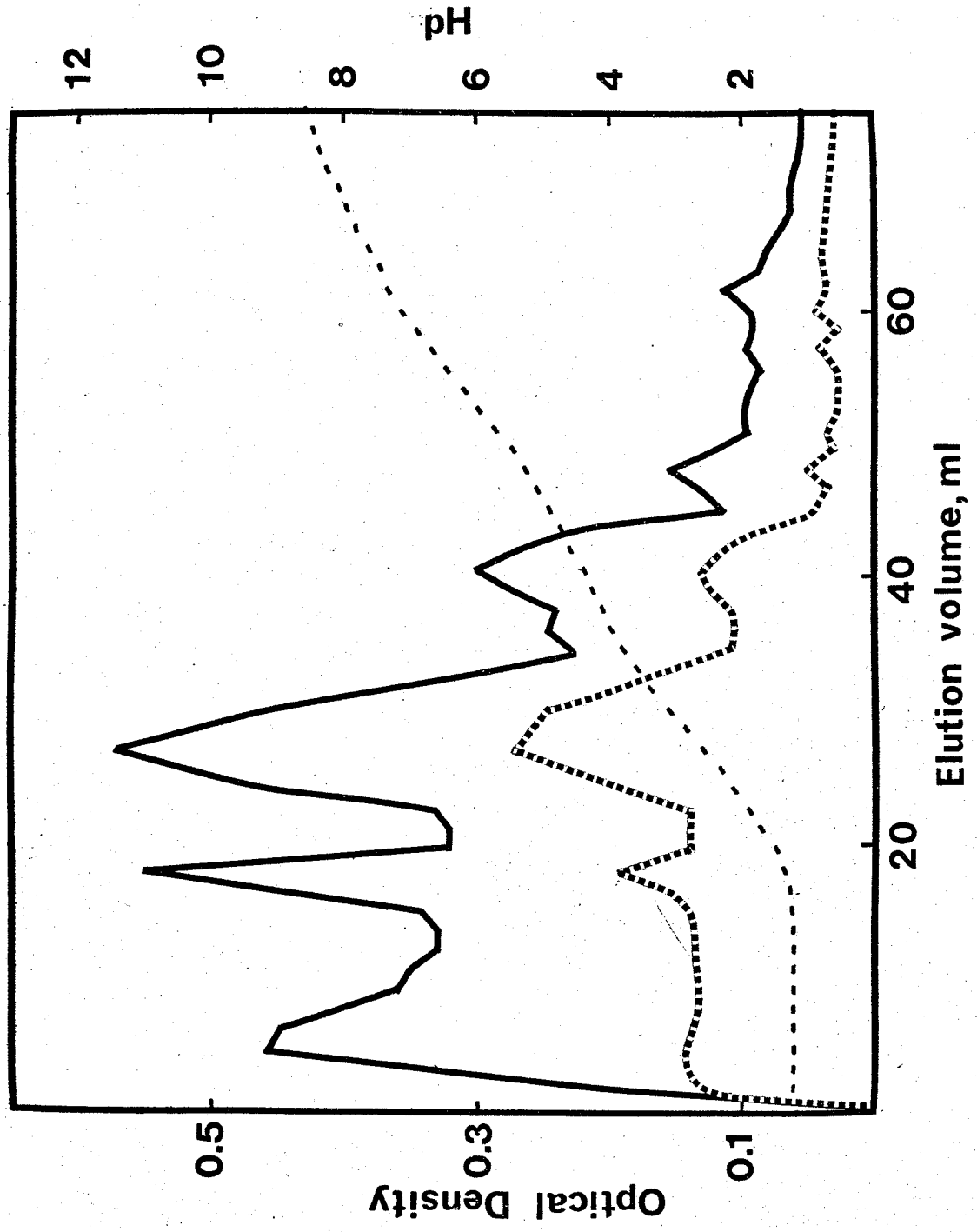
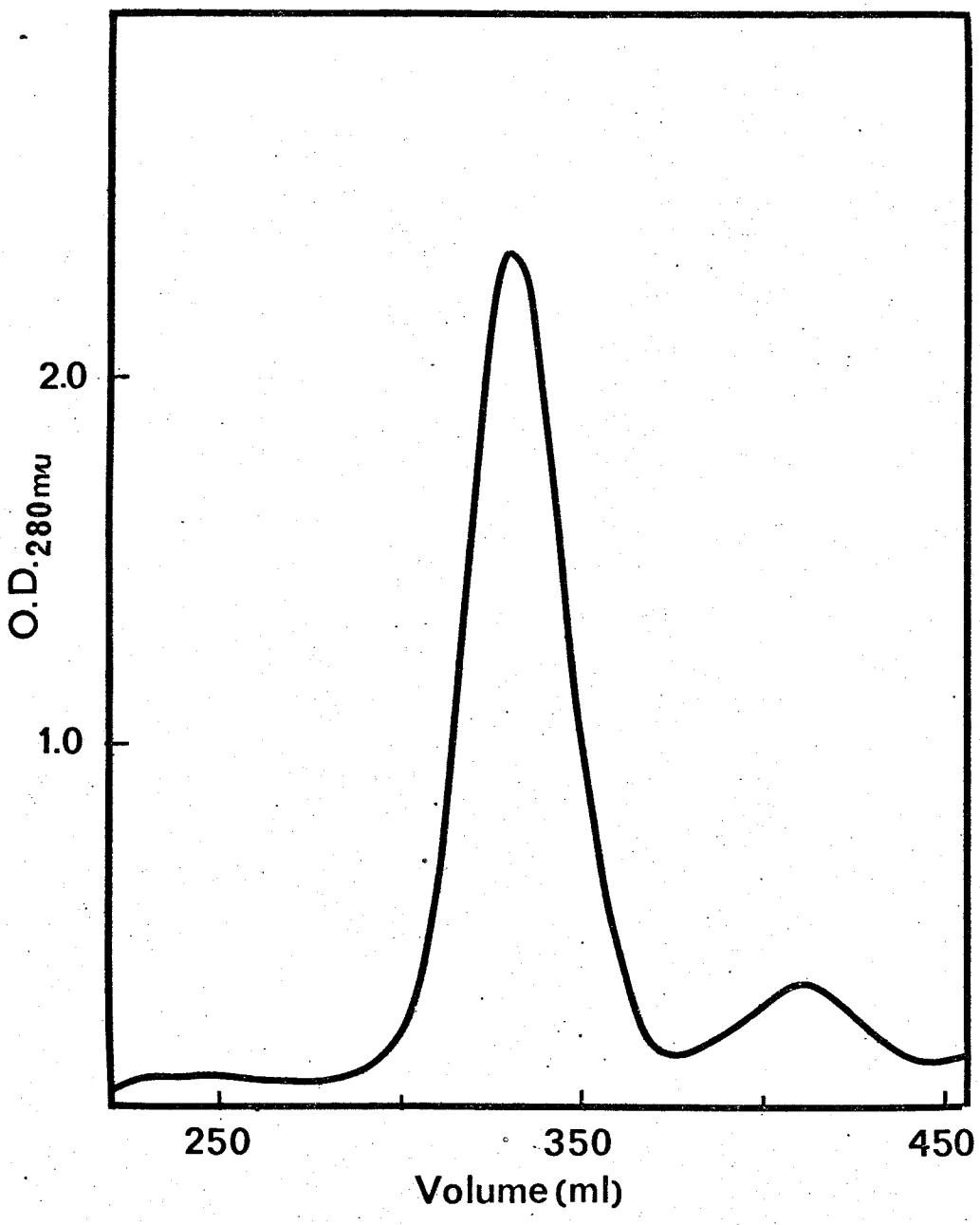

pH
12
10
8
6
4
2
Optical Density
0.5
0.3
0.1
Elution volume, ml
20
40
60

FIGURE 13 Elution pattern of $F(ab')_2$ fragments on a Bio-Gel P-200 column in Tris-HCl buffer (pH 8.0, $\Gamma/2 = 0.1$)


2.0
1.0
O.D.280mu
250
350
450
Volume (ml)

possibility could not be ruled out that the observed peaks were, in fact, due to minor impurities of the DNPI preparation.

Isolation of Fab' Fragments

The elution profile of the pepsin digest of specifically purified anti-DNP antibodies, separated on a Bio-Gel P-200 column, is shown in Figure 13. The major peak which had a sedimentation coefficient of 5.6 S (uncorrected) represents $F(ab')_2$ fragments, while the second peak consists of peptides from the Fc region of the IgG molecule.

Reduction of the $F(ab')_2$ fragments to Fab' yielded, as expected, an ultracentrifugally homogeneous product (Figure 14), sedimenting with a coefficient of 3.6 S (uncorrected).

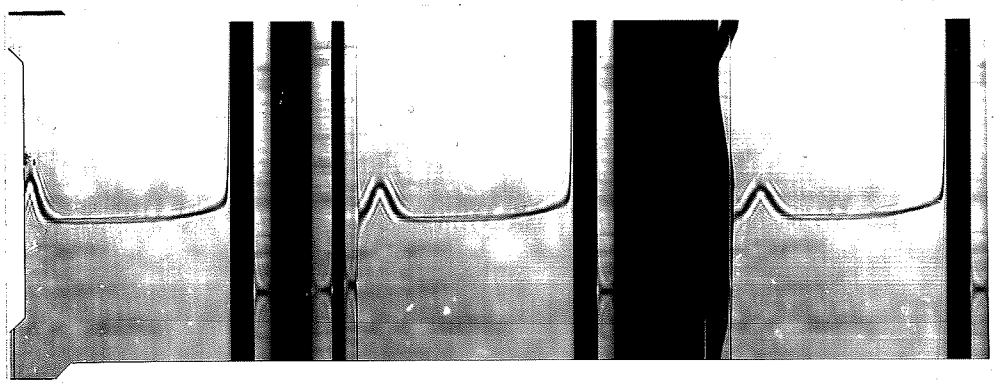
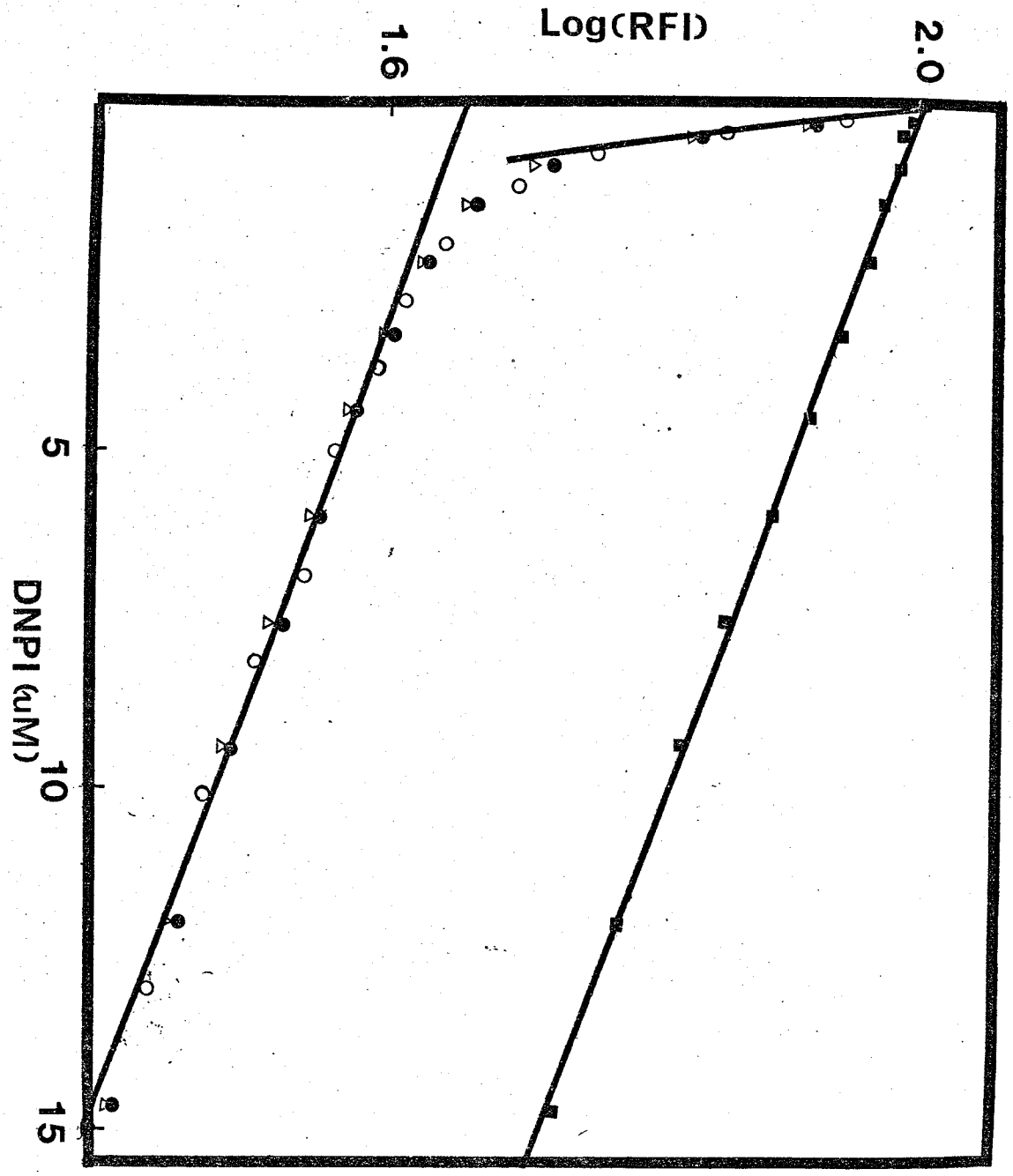


FIGURE 14 Sedimentation pattern of Fab' fragments (2 mg/ml) in 0.01M PBS, pH 7.4, at 59,780 rpm (20°C). Photographs were taken after 25 minutes at 8 minute intervals.

Fluorescence Quenching Studies

Specific binding of DNPI by both intact anti-DNP antibodies and their univalent Fab' fragments was studied. Both preparations were titrated with a large excess of DNPI, in order to determine the values of Q_{max}, which were required for the calculation of binding constants from the appropriate fluorometric titration data. A plot of the fluorescence quenching data which yielded Q_{max} for the reaction of intact anti-DNP antibodies with DNPI, is shown in Figure 15. From this figure, it is evident that DNPI did not quench the fluorescence of normal rabbit IgG ($nR_{\gamma}G$). The linear decrease of log (RFI) for the titration of $nR_{\gamma}G$, as well as that for anti-DNP antibodies at high DNPI concentration was due to antigen attenuation, i.e., the absorption of emitted light intensity by antigen. An extrapolation of the linear portion of the antibody titration curve, yielded a value of log (RFI) = 1.654 and thus a Q_{max} of 55%. The corresponding value for Fab' fragments was 73% (see also Table VIII). While the latter value agrees quite closely with that obtained with ε -DNP-lysine, the former is somewhat lower. A lower Q_{max} value can indeed be expected for the titration of intact antibody, since the fluorescence of the Fc moiety of the molecule should not be quenched by the ligand (McGuigan and Eisen, 1968). It should be pointed out that the slope of the line obtained with $nR_{\gamma}G$, i.e., 0.0175 log (%T) per μM DNPI was used to correct for the attenuation due to DNPI throughout this study.

FIGURE 15 Estimation of Q_{max} for anti-DNP antibodies (50 μg/ml, lower line), by titrating with a high concentration of DNPI (4×10^{-4} M). Titration of control, normal IgG (upper line), shows the extent of DNPI attenuation. Each symbol represents one titration.


Log(RFI)
1.6
2.0
5
10
15
DNPI (uM)

Values for the average intrinsic association constant, K_o^a, and the heterogeneity index, α , were calculated from fluorescence quenching data obtained at higher concentrations of antibody ($\simeq$ 100 µg/ml),titrated with solutions of lower DNPI concentrations ($\simeq 1 \times 10^{-4}$ M). The corrected titration curve (for method of correction see Appendix B) is shown in Figure 16. When the total number of antibody combining sites (Ab_T) was calculated, it was found that DNPI reacted only with 63% of IgG molecules present (the concentration of total IgG was calculated from optical density measurements, at 280 m µ using $E_{1\%}^{1cm} = 15$). This value agree quite well with a value of 70% obtained by Kelly et al. (1971), who titrated the same antibody preparation with ε -DNP-lysine, and who also found that only 70% the molecules of the antibody preparation could be precipitated with HSA-DNP. Similarly, in this study, only 63% of the Fab' fragments reacted with DNPI. The specific titration curve is shown in Figure 17. Since both the antibody and the Fab' solutions contained a yellow impurity, it was concluded that either HSA-DNP which had come off the immunosorbent and/or some 2,4-dinitrophenol, used for elution, remained strongly bound to antibody combining sites of higher affinity. For the purpose of calculating the number of binding sites, only the fraction of IgG molecules, precipitable by specific antigen, was taken into consideration.

From the specific titration curves the fraction of antibody sites occupied (r) and the concentration of free hapten(c) were calculated (Table VII). A plot of these data, in the form recommended by Karush (1962), is shown in Figure 18 for reactions involving both intact antibodies as well as Fab' fragments. The straight lines were fitted

TABLE VII

REPRESENTATIVE FLUOROMETRIC TITRATION OF FAB' FRAGMENTS WITH DNPI AT 25°C

$(H)_T$ [µM]	% Quenching (Q)	r	$\frac{r}{1-r}$	$\log\left(\frac{r}{1-r}\right)$	C [µM]	log C
0.81	63.20	0.50	1.016	0.0068	0.0990	-1.005
1.12	53.48	0.63	1.756	0.2445	0.2125	-0.673
1.47	46.15	0.73	2.811	0.4489	0.4209	-0.376
1.87	40.46	0.81	4.423	0.6457	0.7085	-0.150
2.36	36.16	0.87	6.969	0.8432	1.1185	0.048
2.95	33.80	0.90	9.734	0.9883	1.6591	0.219
3.62	31.68	0.93	14.598	1.1641	2.2934	0.360
4.38	30.87	0.94	17.860	1.2519	3.0387	0.482
5.23	30.08	0.95	22.696	1.3558	3.8657	0.587

FIGURE 16 Specific quenching curve from the titration of anti-DNP antibodies (54 μg/ml) with DNPI (1×10^{-4} M).

Each symbol represents one titration.

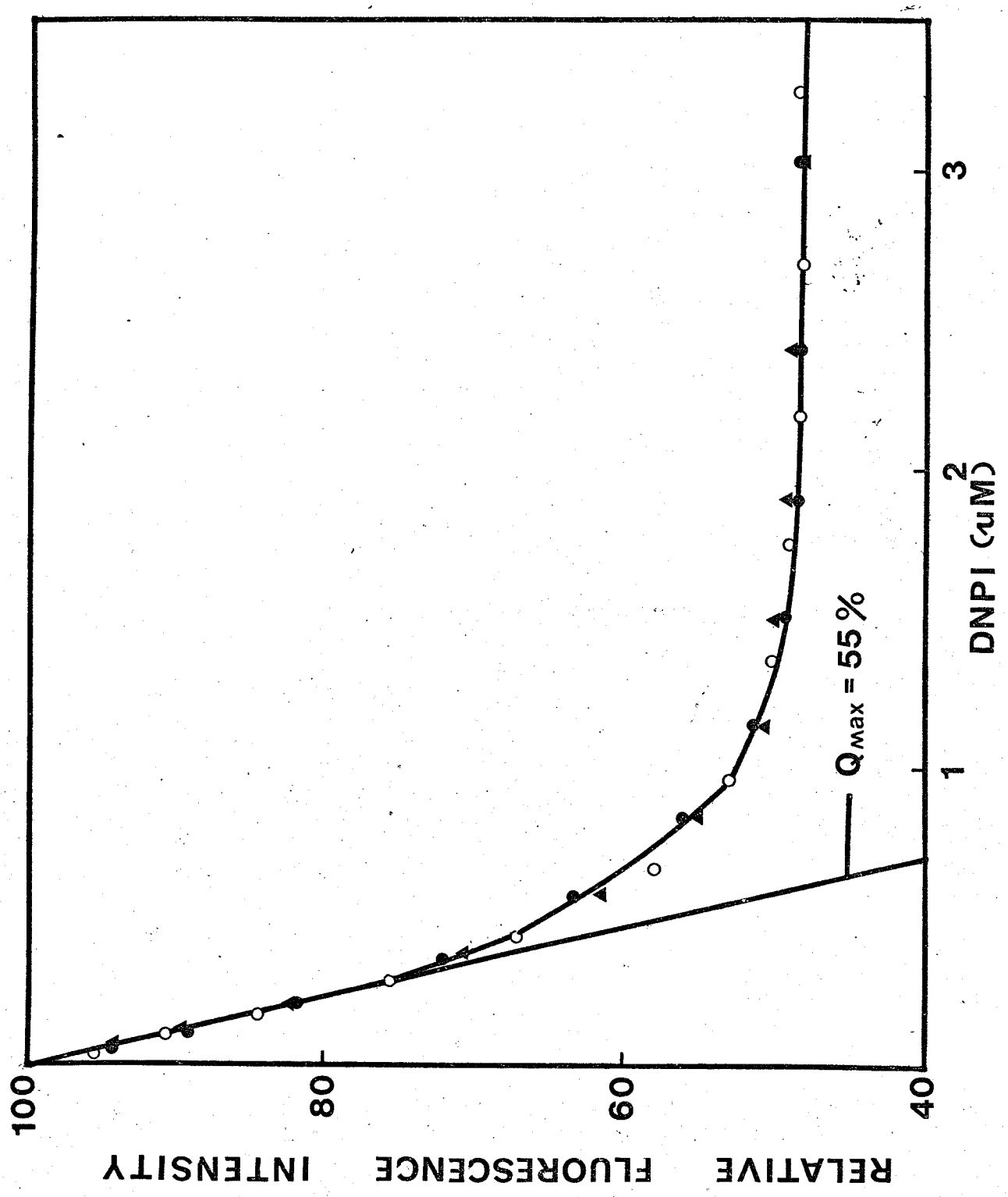

RELATIVE FLUORESCENCE INTENSITY
100
80
60
40
1
2
3
DNPI (μM)
Q_{max} = 55 %

FIGURE 17 Specific quenching curve from the titration of Fab' fragments (78 μ g/ml) with DNPI (1.05×10^{-4} M). Each symbol represents one titration.

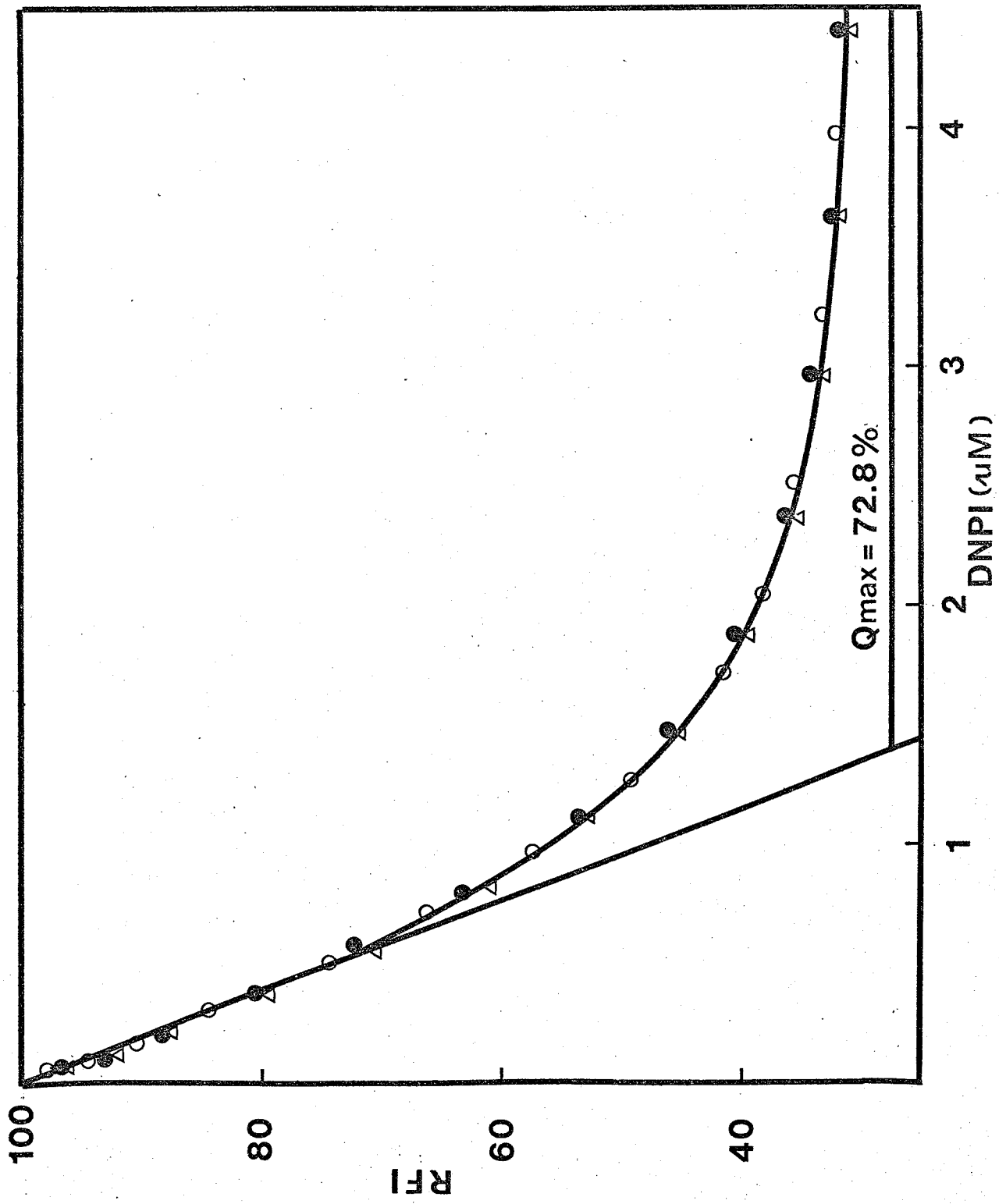
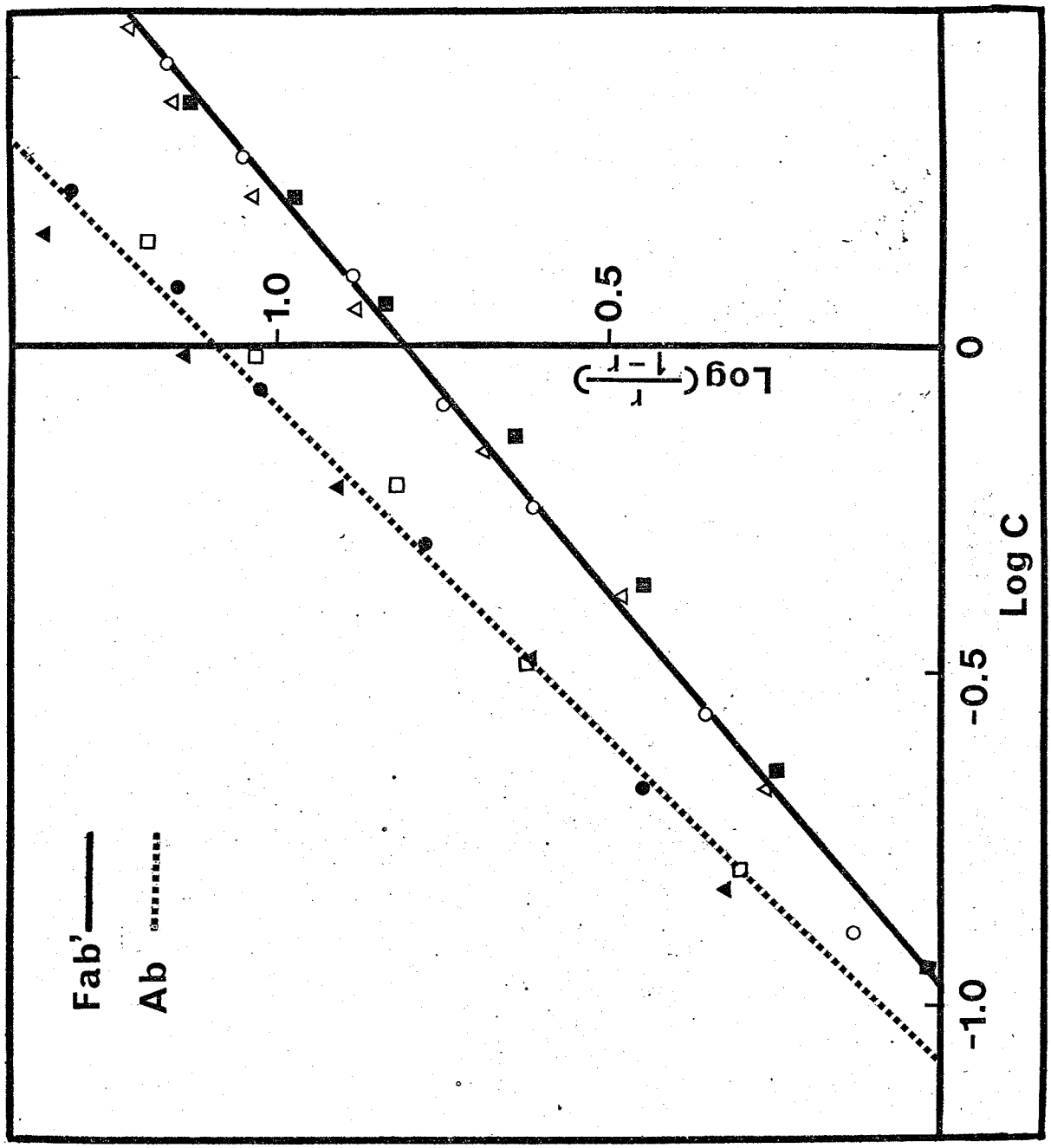

100
80
60
40
RFI
1
2
3
4
DNPI (μM)
Qmax = 72.8%

FIGURE 18 Sips plots for fluorescence titration data at 25°C for DNPI with antibody (dotted line) and Fab' fragments (full line).

Each symbol represents one titration.


Fab'
Ab
1.0
0.5
0
Log (r / 1-r)
-0.5
-1.0
Log C

to the experimental points by the method of least squares. The various binding parameters, obtained from fluorescence quenching data, are summerized in Table VIII.

TABLE VIII

EQUILIBRIUM VALUES FROM FLUORESCENCE QUENCHING STUDIES FOR THE REACTIONS OF DNPI WITH ANTI-DNP ANTIBODIES AND ITS F(ab') FRAGMENTS AT 25°C

Antibody	$\%Q_{max}$	n	α	K_o^a (M^{-1})
Ab	55.0	1.8	0.98	1.20×10^7
F(ab')	72.8	0.9	0.83	0.94×10^7

An inspection of the data listed in Table VIII reveals that the average intrinsic binding constant (K_o^a) for the reaction of DNPI with anti-DNP antibodies is within the range of values obtained for other antigen-antibody systems (see Table II). However, it is somewhat lower than that obtained (2.2×10^7 M^{-1}) when the same antibody preparation was titrated with DNP-lysine (Kelly et al., 1971). Little and Counts (1969), on the other hand, observed slightly higher binding constants for DNPI than for DNP-lysine in the case of guinea pig antibodies elicited by BGG-DNP.

The affinity of Fab' for DNPI was found to be slightly lower (by about 25%) than that of the parent antibody molecules. Tenfold lower affinities in the case of Fab fragments were observed by Levison

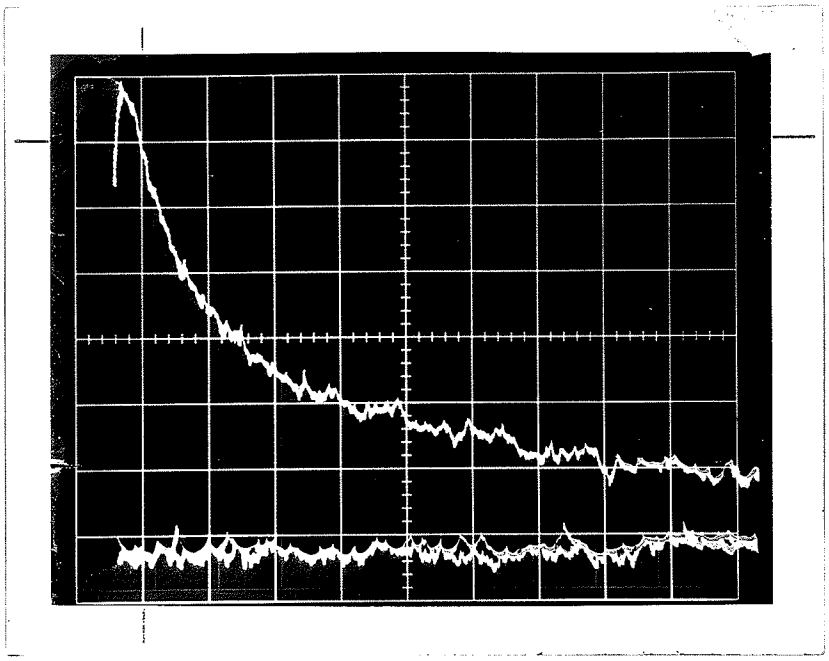
et al. (1970), however, their results were obtained with multivalent antigens and were calculated from rate constants. Such results could actually be expected if bivalent antibody molecules had combined with antigenic determinants on the same antigen molecule or if they were involved in formation of large antigen-antibody complexes. Binding studies on hapten-antibody reactions, when involving Fab fragments, usually yielded affinity constants, which differed only slightly from those involving parent antibody molecules. Thus, Kelly et al. (1971) observed slightly higher binding constants for Fab' fragments, while McGuigan and Eisen (1968) observed both lower and higher values. It should be pointed out that the small differences in binding constants observed in this and the other studies may actually be identical within experimental error, since as pointed out by Kelly et al. (1971), a 5% error in the number of binding sites would lead to a 20% error in the affinity constant. In the present investigation the number of binding sites could be determined only with an accuracy of ± 12%.

A rather surprising finding was that the heterogeneity index, α , for reaction involving both antibody and Fab' was considerably closer to 1.0 than was observed by Kelly et al. (1971) using the same antibody preparation with DNP-lysine as the ligand. The possible significance of this finding will be discussed in Chapter IV.

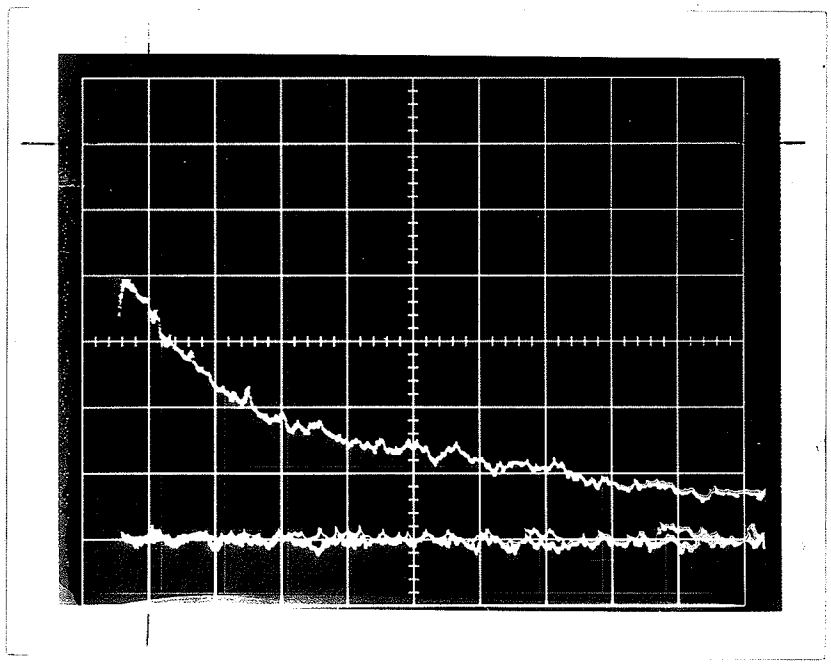
FIGURE 19

(a) Reaction curve for the quenching of fluorescence of antibody (1.1×10^{-6}M) by DNPI (1.3×10^{-6}M) as a function of time at 25°C. Sweep rate: 100 m sec/cm.

(b) Reaction curve for the quenching of fluorescence of Fab' (1.49×10^{-6}M) by DNPI (1.49×10^{-6}M) as a function of time at 25°C. Sweep rate: 50 m sec/cm.



(a)



(b)

Stopped-Flow Studies

Measurements for the rates of reaction of DNPI with anti-DNP antibodies and its Fab' fragments were performed with the aid of a stopped-flow instrument at 25°C. Figure 19 shows actual oscilloscope traces displaying the quenching of antibody (Figure 19a) and of Fab' fluorescence (Figure 19b) by DNPI, with relation to time. The average dead time of 10 msec for the instrument was determined by using a system consisting of BSA: 1N,2,5 S-4DNP. The corresponding oscillographic tracing is shown in Figure 20.

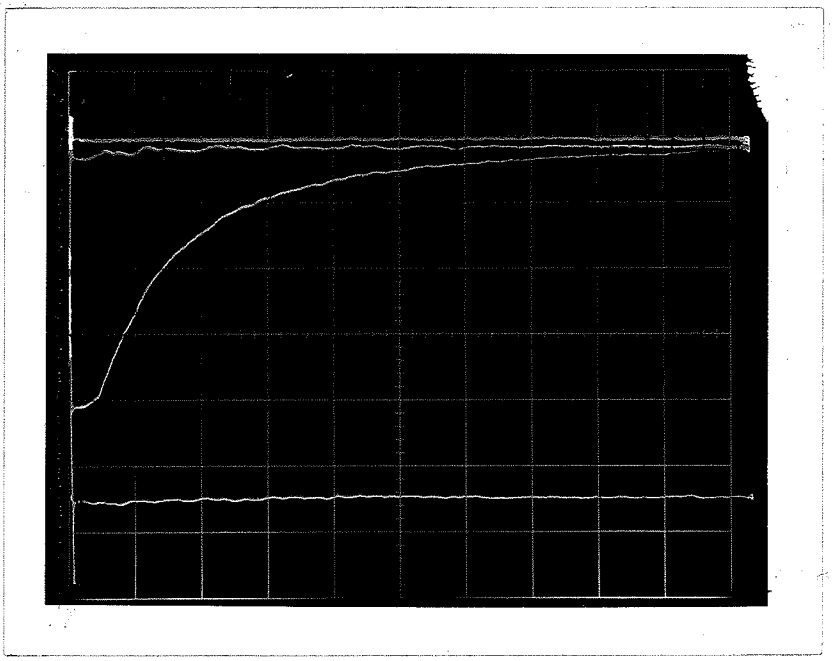


FIGURE 20 Reaction curve at 470 mμ for the BSA: 1N,2,5S-4DNP system in phosphate buffer (pH 6.0, $\Gamma/2 = 0.1$) at 25°C.
Sweep rate: 50 msec/cm.

The kinetic data were analysed according to equation (15). An example of the analysis of the data for the reaction of DNPI with Fab' fragments is shown in Table IX. Data obtained from several reaction curves with varying concentrations of DNPI (4×10^{-5} to 1.3×10^{-6} M), antibody (9×10^{-5} to 1.1×10^{-6} M) and Fab' (1.1×10^{-6} to 1.49×10^{-6} M) were then treated by linear regression analysis. A plot of log $(1-\phi x/1-x)$ versus (mt) is shown in Figure 21 for reactions involving both antibody and Fab'. It is apparent from Figure 21 that the rate data for both intact antibody and Fab' began to deviate from linearity once the reaction had proceeded 40% toward equilibrium. The rate constants calculated from the linear portion for both reaction systems are listed in Table X. The initial forward rate constant (k_{12}) in Table X, for the reaction of DNPI and intact antibody, lies within the range of values commonly determined for antigen-antibody systems (see Table III) and agrees particularly closely with higher values obtained for the insulin: anti-insulin system of Berson and Yalow (1959). However, as could be expected, it is somewhat lower than the corresponding reaction with DNP-lysine (Kelly et al., 1971). It is also interesting to note that the rate constant of association, k_{12}, for the reaction of DNPI with Fab' fragments was somewhat higher than that involving the reaction with the bivalent parent antibody molecule. Using the same antibody preparation and DNP-lysine, Kelly et al. (1971) made a similar observation. In their case, k_{12} for the reaction with Fab' was actually twice as high as that for the intact antibody.

However, Levison et al. (1970) using multivalent antigens such as BSA and ovalbumin, observed that these reacted faster with intact antibodies than with Fab fragments.

The dissociation rate constant (k_{21}), which could not be determined independently by the stopped-flow technique, was obtained from the relationship $k_{21} = k_{12}/K_o^a$, and was found to be 3.92×10^{-1} sec^{-1} for antibody and 5.63×10^{-1} sec^{-1} for Fab' fragments.

Actually, as will be pointed out in the next chapter, this method of calculating k_{21} may not be valid if a two step mechanism is assumed for the reaction between DNPI and anti-DNP antibodies.

Finally, it should be pointed out that a rigorous comparison of the rate constants calculated in this study and those obtained by others for antigen-antibody systems, is difficult to make since it would appear that most authors in calculating rate constants for reactions of antibodies with multivalent antigens used molar concentrations of antibody and antigen, rather than molar concentrations of antigenic determinants and antibody combining sites.

TABLE IX

KINETIC DATA FOR THE REACTION OF FAB' WITH DNPI AT 25°C

Extent of Reaction, (X)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
$\log \left(\frac{1 - \phi X}{1-X}\right)$								
Mean*	0.0206	0.4535	0.0752	0.1126	0.1579	0.2180	0.3071	0.4415
S.D.	±0.0016	±0.0030	±0.0044	±0.0054	±0.0100	±0.0140	±0.0150	±0.0194
(mt)								
Mean*	3.477	8.020	13.915	21.308	31.200	45.878	75.204	134.310
S.D.	±0.327	±0.620	±1.222	±1.614	±1.837	±3.383	±8.409	±26.045

* Mean value of 13 determinations with Fab' concentration varied from 1.1 - 1.4 x 10^{-6}M; and DNPI concentrations varied from 1.16 - 1.49 x 10^{-6}M.

TABLE X

KINETIC DATA FOR THE REACTION OF DNPI WITH ANTI-DNP ANTIBODIES AND FAB' FRAGMENTS AT 25°C.

Antibody and DNPI

Expt. No.	DNPI* (M)	Ab sites (M)	k_{12} ($M^{-1}sec^{-1}$)	k_{21}** (sec^{-1})
1	0.77×10^{-6}	0.93×10^{-6}	4.11×10^{6}	3.42×10^{-1}
2	0.87×10^{-6}	0.98×10^{-6}	5.40×10^{6}	4.50×10^{-1}
3	0.90×10^{-6}	0.93×10^{-6}	4.91×10^{6}	4.09×10^{-1}
4	1.37×10^{-6}	1.08×10^{-6}	4.41×10^{6}	3.67×10^{-1}
MEAN			$4.7 \pm 0.49 \times 10^{6}$	$3.92 \pm 0.47 \times 10^{-1}$

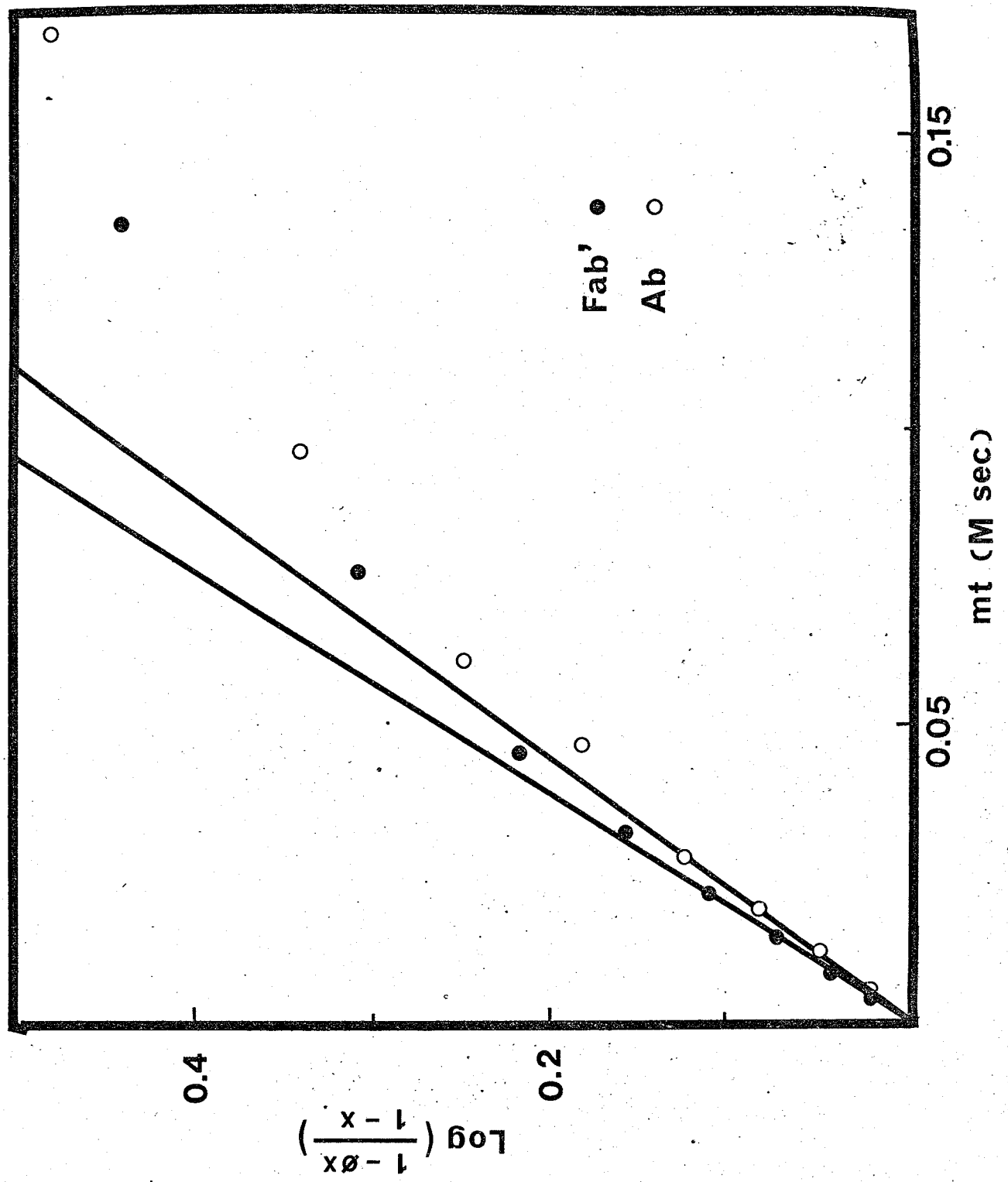
Fab' and DNPI

Expt. No.	DNPI* (M)	Fab (M)	k_{12} ($M^{-1}sec^{-1}$)	k_{21}** (sec^{-1})
1	1.40×10^{-6}	1.49×10^{-6}	5.01×10^{6}	5.32×10^{-1}
2	1.16×10^{-6}	1.49×10^{-6}	5.20×10^{6}	5.53×10^{-1}
3	1.49×10^{-6}	1.11×10^{-6}	6.05×10^{6}	6.42×10^{-1}
4	1.49×10^{-6}	1.49×10^{-6}	4.96×10^{6}	5.27×10^{-1}
MEAN			$5.3 \pm 0.43 \times 10^{6}$	$5.63 \pm 0.54 \times 10^{-1}$

* Expressed in term of DNP groups

** Derived from the relationship $k_{21} = \frac{k_{12}}{K_o^a}$

FIGURE 21 Kinetics of reaction of DNPI with the anti-DNP antibodies and its Fab' fragments at 25°C, plotted according to equation (15). Initial concentration of antibody varied from 0.93×10^{-6}M to 1.08×10^{-6}M; of DNPI from 0.77×10^{-6}M to 1.37×10^{-6}M. Initial concentration of Fab' varied from 1.11×10^{-6}M to 1.49×10^{-6}M, of DNPI from 1.16×10^{-6}M to 1.49×10^{-6}M.


Fab'
Ab
0.15
0.05
mt (M sec)
0.4
0.2
Log ((1 - øx)/(1 - x))

Chapter IV

GENERAL DISCUSSION OF RESULTS

Although hapten-antibody reactions do not appear to differ greatly from antigen-antibody reactions (Singer, 1965), they can at best only serve as model systems for the initial interaction of an antibody combining site with an antigenic determinant. Most haptens used have dimensions smaller than the actual antibody combining site which may encompass sections of the carrier molecule (Worobec et al., 1972b), and the deep sites may actually "swallow up" the hapten molecule (Green et al., 1972). Moreover, the coming together of an antibody molecule with a macromolecular antigen may lead to the secondary interaction of amino acid residues not directly involved in the combining site or the antigenic determinant(s). To investigate this aspect, univalent antigens would be the ideal models since they would not lead to the formation of complex aggregates, which may obscure the observation of the secondary interactions as mentioned above.

Natural occurrence of univalent antigens seems to be rare. Berson and Yalow (1959), on the basis of the kinetic studies, arrived at the conclusion that insulin behaved as a univalent antigen in its interaction with anti-insulin antibodies. Similarly, Worobec et al. (1972a, 1972b) reported that angiotensin I and II appeared to act as univalent antigens. These latter molecules are relatively small but they are immunogenic and are thus considered as antigens, even though they react with antibodies in a manner more similar to that of hapten-antibody than to antigen-antibody reactions.

As pointed out in the introduction to Chapter III, of the partially synthesized univalent antigens, DNP-Insulin appeared to be a particularly useful model antigen for kinetic and equilibrium studies with anti-DNP antibodies, since any results obtained could be compared to those obtained previously by Kelly et al. (1971) for a system consisting of hapten ε-DNP-lysine and an anti-DNP antibody preparation, identical to the one used in the present study.

In spite of the fact that bovine insulin possesses only one lysyl residue per molecule, it was theoretically still possible that insulin aggregates, carrying more than one 2,4-dinitrophenyl group, existed. However, molecular weight determination by gel chromatography, using both column and thin-layer methods, indicated that at concentrations of 1×10^{-4} M (expressed in terms of a molecular weight of 12,000) DNPI existed as a dimer (Figure 6 and 8) and spectrophotometric analysis had revealed that only 0.5 DNP groups

per monomer, or one DNP group per dimer had been coupled. In addition, using identical coupling conditions, Little and Counts (1969) had demonstrated that only lysyl residues of the insulin molecules, and of these only 50%, do react with 2,4-dinitrobenzene sulfonate. The same authors had also observed that it was impossible to separate monosubstituted DNPI from the unsubstituted insulin, thus providing further evidence that the dimerized DNPI preparation (at 1×10^{-4} M) contained most likely equal amounts of monosubstituted DNPI and unsubstituted insulin and therefore this dimerized DNPI was indeed monovalent with respect to the DNP group. It is even quite likely that at the concentrations used in equilibrium and kinetic studies ($\simeq 1 \times 10^{-6}$ M) the DNPI preparation existed in the form of monomers, since Fredericq (1956), Marcker (1960) and Zimmerman et al. (1972) had shown that monomeric insulin is present at concentrations of 0.1 - 0.5 g/l (8×10^{-6} - 4×10^{-5} M). A dissociation of the DNPI preparation into monomers would also have led to the presence of unsubstituted insulin monomers. However, this should not have affected the kinetic and equilibrium data presented in this study in any way, since Little and Counts (1969) had shown that unsubstituted insulin does not cross-react with anti-DNP antibodies. Furthermore, all concentration of DNPI used in the present study were calculated in terms of moles of DNP groups and not moles of insulin.

In discussing the equilibrium and kinetic data obtained in this study, it would be advantageous to compare these data to those obtained by Kelly et al. (1971), using the same antibody preparation and the hapten ε-DNP-lysine.

In Table XI, equilibrium data for reactions involving DNPI and ε-DNP-lysine are compared. While DNPI seems to quench the fluorescence of intact antibody molecules less efficiently than ε -DNP-lysine, both ligands quenched Fab' fragments to the same extent. The observed difference in Q_{max} in the case of intact antibody may be a reflection of steric hindrance to the binding of two DNPI molecules by the bivalent antibody, resulting in a less "intimate" contact between DNP groups and the combining site(s), and thereby making the energy transfer from the tryptophan residues to the DNP group less efficient.

One of the more striking observationsmade in this study was that the heterogeneity index was close to 1 when the antibody (both parent and Fab') was titrated with DNPI. Therefore the intact antibody appeared to be homogeneous when binding with DNPI, yet it exhibited considerable heterogeneity when binding with ε-DNP-lysine. This apparent homogeneous binding energy seemed to be real, since a

TABLE XI

EQUILIBRIUM DATA FOR THE REACTIONS OF ε -DNP-LYSINE AND DNPI WITH ANTI-DNP ANTIBODIES AND IT FAB' FRAGMENTS

Ligand	Antibody	$\%Q_{max}$	α	n	$K_o^a(M^{-1})$	Reference
DNPI	Ab	55.0	0.98	1.8	1.2×10^7	This study
ε-DNP-lysine	Ab	65.3	0.54	2.1	2.2×10^7	Kelly (1970)
DNPI	Fab'	72.8	0.83	0.9	0.94×10^7	This study
ε-DNP-lysine	Fab'	73.4	0.70	-	2.50×10^7	Kelly (1970)

total of six titrations were performed and each separate experiment yielded a value for α which was close to unity. While the reasons for this apparent homogeneity could not be determined experimentally, the following tentative explanation may be put forward: the binding of DNPI by anti-DNP antibodies involved not only the combining site of the antibody and the DNP-lysyl residue of the DNPI, but also the secondary interactions between amino acid residues not directly involved in either the antibody combining site or the antigenic determinant. These secondary, non-specific interactions may have compensated for any differences in affinity of various populations of antibody molecules for the DNP-lysyl residue. Secondary, non-specific interactions may also have taken place between the two insulin molecules, occupying the two combining sites of a bivalent antibody molecule. It is also conceivable that the binding of DNPI was more homogeneous than that of DNP-lysine, because the DNP-lysine residue of the former, due to the presence of the bulky carrier molecule could not adapt as easily to the various antibody combining sites as the free DNP-lysine molecule could. This may also account for the fact that the affinity constant for the binding of DNPI was somewhat lower than that observed by Kelly et al. (1971) for DNP-lysine. Although a difference in binding affinity for DNPI was observed between intact antibodies and Fab' fragments, this is, as mentioned previously, not considered significant since Kelly et al. (1971) had calculated that a 5% error in the number of antibody combining sites would lead to a 20% error in the average intrinsic association constant. In the present study, an error of 12% was

calculated for computing K_o^a values, without taking into consideration possible errors in the determination of the total number of antibody combining sites.

While data obtained from binding studies did reveal some differences between the interactions of DNPI and DNP-lysine with the same antibody preparation, they could provide no direct answers as to why this was so, particularly, since equilibrium constants are only a measurement of free energy differences between products and reactants. Details as to the mechanism of the reaction are best obtained from kinetic studies.

An inspection of the oscillographic tracings obtained from stopped-flow measurements of the reaction between DNPI and anti-DNP antibodies reveals immediately the biphasic nature of the reaction. There appeared to be an initial rapid decrease in the fluorescence intensity followed by a slower approach to equilibrium. Kelly (1970) using DNP-lysine as the ligand did not observe this second part of the reaction. When the rate data are plotted according to Day et al. (1963) (Figure 21), the difference between the DNPI and DNP-lysine systems becomes even more apparent. In the DNPI system, a linear relationship was only observed up to a point at which only 40% of the reaction had gone to completion ($x = 0.4$) (Figure 21). On the other hand, in the DNP-lysine system (Kelly, 1970) linearity was observed even when the reaction was 80% completed. Day et al. (1963) had interpreted deviation from linearity in terms of the heterogeneity of the antibody. However, this argument cannot be invoked to explain the data presented in this study, since the reaction with DNPI

appeared to be more homogeneous than that with DNP-lysine. Therefore, the following reaction mechanism is proposed for the reaction of DNPI with anti-DNP antibodies

$$\underset{(I)}{Ab + Ag} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \underset{(II)}{AbAg^{*}} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \underset{(III)}{AbAg} \qquad (17)$$

Here, the initial fast combination of antibody with antigen, is followed by a more slow rearrangement of the complex due, at least partially, to the interaction of amino acid residues not directly involved in the antibody combining site or the antigenic determinant. A similar two-step mechanism has been proposed by Levison et al. (1970) for the reaction of bivalent antibody with multivalent antigens. However, these authors interpret the mechanism by assuming first a loose encounter pair followed by a rearrangement of the complex. They consider the second step to contribute the major portion of the free energy of reaction. Moreover, they only propose this mechanism for reactions involving bivalent antibody in the present of non-chaotropic ions, while in the present study the biphasic nature of the reaction was observed for both bivalent antibody and univalent Fab' fragments.

From the initial linear rate data, a rate constant (k_{12}) for the initial specific combination of antigen with antibody was calculated. In Table XII, these rate constants are compared with those obtained by Kelly et al. (1971). It is obvious from this table that the forward rate constants (k_{12}) for reactions involving DNPI are somewhat smaller than those for reactions in which ε -DNP-lysine participated. In general, this phenomenon could be attributed to

TABLE XII

RATE CONSTANTS FOR REACTIONS OF DNPI AND ε-DNP-LYSINE WITH ANTI-DNP ANTIBODIES AND THEIR Fab' FRAGMENTS

Ligand	Antibody	k_{12} ($M^{-1}sec^{-1}$)	k_{21}^{a} (sec^{-1})	Reference
DNPI	Ab	$4.70 \pm 0.49 \times 10^{6}$	$3.92 \pm 0.47 \times 10^{-1}$	This study
ε-DNP-lysine	Ab	$1.10 \pm 0.20 \times 10^{7}$	5.1×10^{-1}	Kelly(1970)
DNPI	Fab'	$5.30 \pm 0.43 \times 10^{6}$	$5.63 \pm 0.54 \times 10^{-1}$	This study
ε-DNP-lysine	Fab'	$2.00 \pm 0.20 \times 10^{7}$	8.0×10^{-1}	Kelly(1970)

a - Derived from the relationship: $k_{21} = \frac{k_{12}}{K_o^a}$

the two reasons as given at the end of Chapter II. These are: (1) If the rate of the antigen-antibody reaction is diffusion controlled, then the bigger antigen molecule diffuses slower toward the antibody combining sites as compared with the smaller ε-DNP-lysine molecule; (2) Steric hindrance on the part of the antigen molecule due to partial blocking of the antigenic determinant by amino acid residues near the DNP-lysyl residue.

For a ligand of spherical symmetry diffusing into a hemispherical combining site, in the absence of charge effects, the diffusion controlled rate constant can be calculated following the suggestion of Alberty and Hammes (1958):

$$k_{12} = \frac{2N\pi}{1000} R_{1,2} \times D_{1,2} \qquad (18)$$

where

N = Avogadro's number

$R_{1,2}$ = Reaction radius of the two reacting species

$D_{1,2}$ = Sum of the diffusion coefficients of the reacting molecules

Using this equation for the DNPI: anti-DNP antibody system, a rate constant of $7.4 \times 10^{8} M^{-1} sec^{-1}$ was calculated; where the reaction radius was taken as 10×10^{-8} cm, being the longitudinal dimension of anti-DNP antibody combining site (Hsia and Little, 1971), and the diffusion coefficients as 15.5×10^{-7} $cm^{2} sec^{-1}$ for DNPI (Fredericq, 1956), and $4.1 \times 10^{-7} cm^{2} sec^{-1}$ for rabbit IgG molecules (Edsall, 1953). This value for the rate constant is about 2 orders of magnitude larger than the experimental rate constants obtained in the present study. Differences of the same order of magnitude between calculated and observed rate constants were observed by Froese (1962) and Day et al. (1963).

It should be pointed out that the above calculation of a diffusion controlled reaction particularly in the case of an antigen antibody reaction is at best an approximation, since in the case of the antigen only a small fraction of its surface (the DNP-lysyl residue) can react with the antibody combining site. Thus, a great number of collisions will not lead to the formation of antigen-antibody complexes.

With reference to Table XII, it should be mentioned that if the reaction mechanism represented by relationship (17) really holds, a calculation of the rate constant of dissociation (k_{21}) by the method indicated (footnote a of Table XII) may not have been

permissible, since this equation holds only for single-step reversible reactions. By the same token, it can be argued that equation (16) was not applicable to the calculation of kinetic data in this study, particularly since K_o^a represents the equilibrium constant for the overall reaction and not just that between states I and II of equation (17). However, if k_{12} is much greater than k_{21} in the initial combination of antigen and antibody, it should be possible to calculate k_{12} assuming a non-reversible reaction of the type

$$Ab + Ag \longrightarrow AbAg \qquad (19)$$

for the initial phases of the reaction between DNPI and antibody. Indeed, such treatment of the rate data yielded rate constants which were practically identical to k_{12} of Table XII.

A comparison of the rate data obtained with intact antibody and Fab' fragments shows that the rate constant (k_{12}) for the reaction of Fab' fragments with DNPI is somewhat larger than that involving the parent antibody. Using the same antibody preparation and DNP-lysine, Kelly et al. (1971) had made a similar observation. However, they observed a larger (two-fold) difference. In an attempt to explain this difference, these authors had proposed that the combining sites of an intact antibody molecule are directed inward, thus making them less accessible on a bivalent antibody molecule than they would be on an univalent Fab' fragment. If this were indeed the case, one could expect even larger differences in rate constants if DNPI is used instead of the smaller DNP-lysine molecule. This was not observed in the present study. Further experiments would be required to settle this problem.

SUMMARY

(1) A univalent antigen, DNP-Insulin, was prepared by coupling a 2,4-dinitrophenyl (DNP) group onto the sole lysyl residue at position 29 of the B chain of bovine insulin. Spectrophotometric analysis revealed that only one DNP group had been introduced per insulin dimer. Column and thin-layer gel filtration chromatography experiments in 0.01M PBS, pH 7.4 established that the DNP-Insulin existed as dimers (M.W. = 12,000) at a concentration of 1×10^{-4}M.

(2) The affinity constant (K_o^a) for the reaction of anti-DNP antibodies and their univalent Fab' fragments with DNP-Insulin as studied by fluorescence quenching, was found to be about one half of that for the corresponding reaction involving the hapten ε-DNP-lysine.

(3) The binding of DNP-Insulin by both intact antibody and Fab' fragments appeared to be more homogeneous than the binding of ε-DNP-lysine by the same antibody preparation.

(4) Kinetic experiments performed with the stopped-flow technique revealed that both bivalent antibody and Fab' fragments reacted with DNP-Insulin in a "biphasic" manner: an initial rapid drop-off in fluorescence was followed by a slower approach to equilibrium.

(5) Rate constants of association for the reaction of DNP-Insulin with intact antibody and their univalent Fab' fragments were found to be $4.7 \times 10^6 M^{-1} sec^{-1}$ and $5.3 \times 10^6 M^{-1} sec^{-1}$, respectively. Both rate constants were lower than those observed by others for reactions involving DNP-lysine.

APPENDIX A

An example for calculating the degree of substitution of insulin by DNP

Extinction coefficients:

Insulin : $E^{1\%}_{1cm} = 10.0$ at 280 mμ

ε-DNP-lysine : $E^{M}_{1cm} = 1.74 \times 10^4$ at 360 mμ

The ratio of the optical density of ε-DNP-lysine at 363 mμ and 280 mμ

$$\left(\frac{\text{O.D. } 363}{\text{O.D. } 280} \right) = 2.89$$

Molecular weight of insulin = 5750

Suppose in a particular DNPI preparation, the O.D. at two different wavelengths were

$O.D._{280} = 5.52$

$O.D._{360} = 5.49$

Therefore,

$O.D._{280}$ due to DNP-lysine $= \frac{5.49}{2.89} = 1.89$

True $O.D._{280}$ due to insulin $= 5.52 - 1.89 = 3.63$

Molarity of DNP-lysine $= \frac{5.49}{1.74 \times 10^4} = 3.14 \times 10^{-4} M$

Molarity of Insulin $= \frac{3.63 \times 10}{10 \times 5750} = 6.2 \times 10^{-4} M$

and DNP/insulin $= \frac{3.15 \times 10^{-4}}{6.2 \times 10^{-4}} = 0.5$

APPENDIX B

The treatment of data for a typical fluorometric titration experiment

Fab' = 78 μg/ml

DNPI = 1.03×10^{-4} M

Total volume DNPI added (ml)	0.0000	0.0070	0.016	0.030	0.060	0.090
DNPI concentration [μM], (H_T)	0.00	0.35	0.82	1.47	2.95	4.38
Relative Fluorescence Intensity (RFI)	93.0	74.0	57.2	41.0	28.5	24.5
Blank Corrected RFI	91.0	72.0	55.2	39.0	26.5	22.5
Dilution Corrected RFI	91.0	72.2	55.6	39.5	27.2	23.5
Normalized RFI	100.00	79.40	61.14	43.47	29.97	25.82
Log RFI	2.000	1.899	1.786	1.638	1.476	1.411
Log (DNPI attenuation)	0.000	0.007	0.014	0.026	0.052	0.078
Corrected RFI	100.00	80.54	63.20	46.84	34.80	32.24

Sample Calculation:

The DNPI concentration, on addition of 0.0070 ml DNPI to 2.0 ml of antibody solution,

$$(H)_T = \left(\frac{0.007}{10^3} \times 1.03 \times 10^{-4} \right) \times \left(\frac{10^3}{2.007} \right) \times 10^6$$

$$= 0.35 \ \mu M$$

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

= 74 - 2 = 72% T

APPENDIX B (Continued)

Dilution Corrected RFI $= 72 \times \frac{2.007}{2.000} = 72.2\%$ T

Normalized RFI $= 72.2 \times \frac{100.0}{91.0} = 79.4\%$ T

The DNPI attenuation coefficient was estimated to be 0.0175 log (% T) per μM of DNPI

DNPI attenuation correction:

log (normalized RFI) + DNPI attenuation

$= 1.899 + 0.0175 \times 0.35$

$= 1.9060$

Corrected RFI = antilog 1.9061

= 80.54 % T

The corrected RFI was then plotted as a function of DNPI concentration, $(H)_T$, to yield the specific quenching curve (Figure 16).

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