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Albumin-Dye and Antibody-Hapten Interactions

Chemistry Ph.D.

A study was made of the interactions of two azo-dye pH indicators (and related molecules) containing the 2, 4-Dinitrophenyl determinant group with Bovine Serum Albumin (BSA) and rabbit antibodies specific for the 2, 4-Dinitrophenyl group. Both dyes were found to undergo spectral shifts when binding to these proteins under suitable conditions. The equilibrium constant for the reaction of one of these dyes, 1-Napthol 4-(2, 4-dinitrophenylazo) 2-Sulfonic acid with BSA was evaluated and the effect of pH and inhibitors on the system studied. The kinetics and equilibrium aspects of the interaction of the above dye and of 1-Napthol 2-(2, 4-dinitrophenylazo) 3, 6-disulfonic acid with rabbit antibodies was also studied using the temperature jump technique. The results obtained were discussed in terms of the effects of the different structures of these two dyes on the reactions observed.

ALBUMIN-DYE AND ANTIBODY-HAPTEN INTERACTIONS

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by

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

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TO MY PARENTS

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PART A

THE PROPERTIES OF ANTIGENS AND ANTIBODIES

In vertebrates, recovery from a disease caused by an infectious organism is often found to leave the animal in a state of increased resistance to reinfection. The occurrence of this so-called "immune" state usually coincides with the appearance of proteins, which by definition are called antibodies, in the animal's serum, while the substance that elicits their formation is called an antigen. The early studies of antibody-antigen combinations were complicated by the large size and complexity of these materials. However, early in the 20th century, Landsteiner found that antibodies could be produced to small, well-defined organic molecules, which he called haptens, if they had been chemically coupled to a protein before injection (1). This discovery was responsible for a breakthrough in the understanding of the reactions of antibodies and antigens on a molecular basis, and is therefore considered to mark the beginning of the science of immunochemistry.

Antigens

The ability of a substance to be antigenic in a given animal depends on two general properties, i.e., first, the material must be foreign to the animal, and second, it must be of sufficiently high molecular weight. However these factors are not well enough understood at the present time to allow any "a priori" prediction of a material's antigenicity.

Many different types of materials are known to be antigenic; these range from naturally occuring serum proteins, red blood cells, polysaccharides, (2) and lipids, (3) to synthetic molecules such as polyvinyl pyrrolidone (4) and polyamino acids (5), (6). In contrast to a hapten, which is only antigenic when attached to a large carrier molecule, these latter synthetic materials can elicit an antibody response when injected by themselves, a property that allows a systematic approach to the study of the factors that are involved in antigenicity. Studies with synthetic antigens have allowed a determination of the lower limits of molecular weight of an antigenic material. For example an ε-dinitrophenyl L-lysine nonapeptide has recently been found to be antigenic in guinea pigs (7). Similarly, experiments with polypeptides of different structure and charge have shown that a material's antigenic sites must be readily accessible and not hidden in the molecules "interior" (8). Moreover the use of uncharged polypeptides (9) and of polypeptides possessing either a net positive or negative charge (10), has indicated that (A) the antigen does not have to be charged to cause a specific antibody response and (B) that the net charge of an antibody molecule will be opposite to that of the eliciting antigen. The latter has been interpreted as being an important factor in the stabilization of the interaction between charged antigens and their homologous antibodies (11).

Antibodies

Antibody activity has been found to be associated with three main types of globular proteins whose most important physical properties are given in Table I.

Table I (12)

Immunoglobulin type	I_gG	I_gA	I_gM
Electrophoretic mobility at pH 8.6 (10^{-5} cm^2 V^{-1} sec^{-1})	-0.6 to +3	+1.2 to +3.6	+2
Sedementation coefficient (S)	6.6	6.6 and 12	19
Molecular weight (approximate)	150,000	150,000 and 400,000	900,000

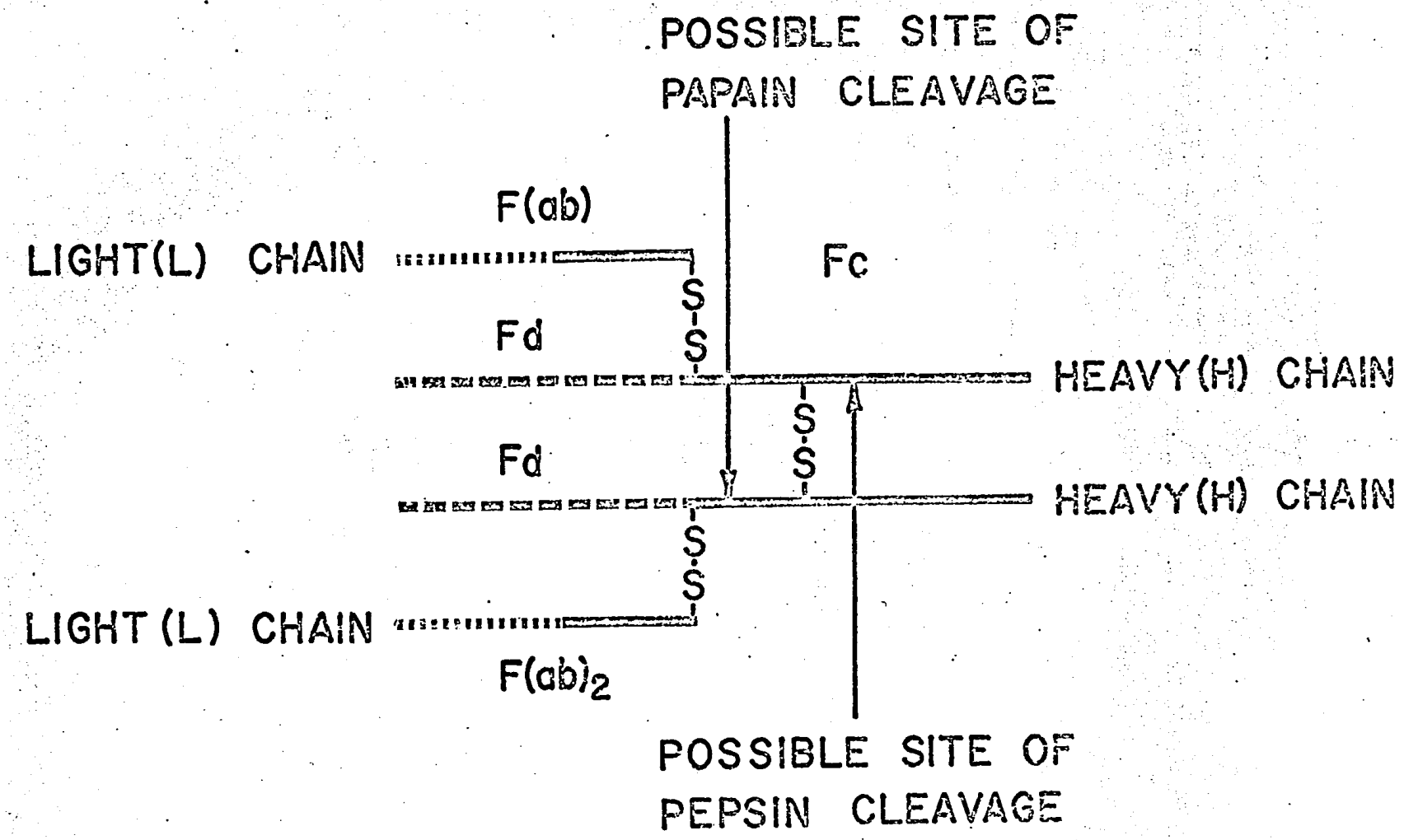
It is because of this complexity that the term immunoglobulin (13) has been suggested to refer to all those globulin molecules possessing antibody activity, as well as to other proteins which may have no (known) antibody activity, but which are structurally related to antibodies. The properties of the immune globulins of all mammals are very similar. However, for reasons of brevity and primarily because of the widespread use of these globulins in immunochemical investigations, the following discussion will be mainly confined to the properties of the rabbit I_gG molecule.

I_gG molecules have been shown to contain two classes of peptide chains, which can be separated by gel chromatography under acid conditions or in the presence of urea after reduction (and alkylation) of disulfide bonds (14, 15). Each molecule consists of two heavy (H) chains (M.Wt. $\sim$ 50,000) and two light (L) chains (M.Wt. $\sim$ 20,000) (16, 17) and has been represented (18) by the schematic diagram shown in Fig. 1.

The number of disulfide bonds between each heavy and light chain has been well established (15). However, uncertainty exists as to the number of these bonds between the heavy chains. Thus, it has been found that about one half of an I_gG preparation could be separated into

Figure 1

Schematic diagram of the I_gG immunoglobulin


POSSIBLE SITE OF
PAPAIN CLEAVAGE
F(ab)
LIGHT(L) CHAIN
Fc
S
S
Fd
HEAVY(H) CHAIN
S
S
Fd
HEAVY(H) CHAIN
S
S
LIGHT (L) CHAIN
F(ab)2
POSSIBLE SITE OF
PEPSIN CLEAVAGE

equivalent half molecules by reduction of one disulfide bond, while separation of the remainder of the molecules present required the reduction of 3 disulfide bonds (19). This phenomenom has been interpreted in terms of a heterogeneity of I_gG molecules with respect to the number of these disulfide bonds per molecule or to the lability of this disulfide bond in different molecules (19); however the possibility that the apparently low number of inter-H-chain disulfide bonds found in half the antibodies may have been due to disulfide interchange during cleavage was not ruled out (12).

As has been mentioned above, dissociation of the I_gG molecule cannot be effected without recourse to low pH or reagents such as urea. It is, therefore, obvious that the peptide chains are held together by non-covalent forces as well. These have been postulated to be mainly hydrophobic in nature (20,21) and in the case of the forces between H chains, to be located in the F_c portion of the molecule [Fig. 1].

The I_gG molecule can also be split into smaller fragments by the action of proteolytic enzymes (acting in concert with reducing agents) as indicated in Fig. 1. Papain splits the molecule into three fragments, two F_{ab}* and one F_c. The two F_{ab} fragments each possess one of the antibody's two binding sites and are otherwise identical having a range of electrophoretic mobility that is related to the parent I_gG molecule from which they are derived (22). The third fragment, F_c (or fragment III) obtained from papain digestion has no antibody site. It is of different character from the F_{ab} fragments and has the unusual property of being easily crystallized, which would indicate that this part of the I_gG molecule is relatively homogeneous.

* Two types of F_{ab} fragments can be obtained by ion exchange chromatography on, for example, carboxyl methyl cellulose (22). Those with higher electrophoretic mobility at neutral pH are known as type I and those with lower mobility as type II.

The action of pepsin is similar to that of papain; however, this enzyme reacts in such a manner as to leave the section of the H chains containing the disulfide bond intact (Fig. 1). A reducing agent can split the divalent $F(ab')_2$ fragment produced into two univalent ones and these can be reoxidized to the divalent form of the antibody (23). This enzyme also causes more extensive degradation of the inactive fragment (corresponding to F_c of papain), splitting it into at least three groups of peptides (12).

The most surprising result of the various enzymatic studies that have been performed is the fact that the fragments produced maintain their ability to resist further degradation as well as the integrity of the antibody binding sites. This has been suggested to mean that these enzymes act at limited sites which are contained in flexible portions of the H chain, and that the three molecular fragments (F_{ab}, F_c) are compact globular entities that are not susceptible to enzymatic attack (24, 25). This conclusion, (regarding the tripartite globular nature of the molecule) has also been supported by measurements of the depolarization of fluorescence of dyes attached to the I_gG molecule (26, 27) and by electron micrographs (28).

The conformation of the intact I_gG molecule has also been studied by low angle X-ray scattering (29, 30) and by hydrodynamic methods (24). These studies had suggested that the molecule was cylindrical in shape with major and minor axes of about 250 and 31 Å. These results may have to be reinterpreted, however, in the light of the discoveries quoted above i.e. ref's 24-28. Attempts have also been made to obtain details of the tertiary structure of the molecule by measurements of optical rotatory dispersion (O.R.D.) spectra, however, the only quantitative results

available predict that the I_gG molecule has less than 10% α-helical content (29, 31, 32). In general, this approach has, as yet, not been very successful due to an incomplete theoretical basis for the understanding of O.R.D. spectra of molecules that are largely non-helical. Nevertheless, one important observation, namely that O.R.D. spectra of rabbit antibodies (to the 2, 4-dinitrophenyl determinant group) differ from the other immunoglobulins present in the animal, has been made (31). This effect was found to be due to the properties of the F_{ab} fragment as the O.R.D. spectra of the antibody F_c fragments, were the same as that of the other I_gG molecules present. Although it was not possible at the time to correlate the spectra obtained with the structure of the fragments, this result indicates that significant differences may be found in the structures of antibodies of different specificity.

Another question related to molecular conformation is the relative location of the two antibody active sites demonstrated to exist in the I_gG molecule (33, 34). These have been suggested to lie at opposite ends of the molecule (35); however, recent electron micrographs have indicated that the positions of the sites, and thus the conformation of the antibody, may depend on the number of antibody valencies occupied (28). Thus, when one site of antibody to ferritin, had reacted with ferritin antigen the antibody appeared to exist as a compact structure about 100 Å long. However, when two antigens were bound the molecule seemed to open up about a hinge point to produce a structure about twice as long. This observation has also been supported by other conformational studies of the I_gG molecule (26, 27, 29) which also led to the interpretation of the molecule's behavior in terms of a variable structure.

The Purification of Antibodies

The procedures used for the isolation of antibodies may be divided into two groups; A) non-specific methods which fractionate antisera on the basis of physicochemical properties common to both antibody and non-antibody globulins, and B) specific methods in which advantage is taken of the specific combination of antibodies and homologous or related antigens.

Of the non-specific methods the most crude is probably that of precipitation, using salts (36), organic solvents (37), or complexing agents (38). Electrophoretic methods, on supporting materials (39) or in solution (40), are probably the most gentle of the non-specific methods, while ion exchange chromatography on various cellulose derivatives (41) has proven very satisfactory but may subject the proteins to mild denaturation during adsorption and desorption from the ion exchanging sites.

In specific purification methods four steps are involved, i.e. I) formation of antibody - antigen complexes, II) isolation of these complexes from other contaminants, III) dissociation of the complexes, and IV) separation of the antibody and antigen. Of these step I will obviously be similar in most systems; step II would be expected to be relatively simple, eg. washing of antigen-antibody precipitates would be an effective way of isolating these complexes in a precipitating system; and the most difficulties would be expected to arise in performing steps III and IV. Various approaches have been adopted for the specific purification of antibodies (42). In many cases these are applicable to only one antigen-antibody system as the method is tailor-

made to accommodate the unique properties of the antigen or antibody involved. However, one general approach that deserves mention is the use of immunosorbents, which consist of insoluble supports to which the desired antigen (§), or hapten, can be chemically attached. Various methods are used to elute antibodies from immunosorbents, eg. low or high $_{p}H$, high salt concentrations or excess of hapten. The latter appears to be the most gentle method; the eluted antibody can then usually be readily separated from the hapten by dialysis or chromatographic procedures. The versatility of immunosorbents has been demonstrated with their application to the purification of antibodies to many different determinants, (42) and in general it would seem that their use provides the most efficacious method of obtaining pure antibodies in high yield.

The Nature of the Antibody Combining Site

Two general theories have been proposed to explain the structural basis of antibody specificity. One is that an antibody with a given amino acid sequence can be folded in different ways and that each folding pattern corresponds to a different specificity. The second view is completely different and maintains that it is the amino acid sequence alone that dictates an antibody's specificity and configuration (43). Strong support for the latter idea has been obtained from studies that have shown that renatured antibodies to ribonuclease (44) and to the 2, 4 - dinitrophenyl determinant group (45) could regain their antigen binding capacity after complete reduction of disulfide bonds and unfolding to a random coil configuration. Attempts have also been made to substantiate this result by determining the amino acid content of (specifically

purified) antibodies of different specificity. To this date antibodies to nine different antigenic determinants have been studied and small but significant differences in amino acid content found between them (46). However, it is possible that these differences do not occur in the actual combining site region, as it has been noted that large differences were found in the peptide maps of antibodies of the same specificity prepared in different rabbits of the same allotype (Disc. ref 46). It thus appears that an unequivocal answer to this question will only be obtained by X-ray analysis of pure antibody crystals. As yet this has not been achieved.

Experiments with antibodies of different specificities have allowed a number of generalities to be made concerning the relation of the properties of the active site to those of the homologous (antigen or) hapten. For example, the implication of amino groups in the binding sites of anti-benzenearsonate antibodies (47), and of carboxylate groups in the active sites of antibodies directed against a positively charged hapten (p-azophenyl trimethylammonium), (48) indicates that an antibody site will probably contain an amino acid of opposite charge to that of the antigenic determinant group. The finding of tyrosine in the combining sites of antibodies directed towards neutral, positively and negatively charged haptens (49) has been interpreted as meaning that the function of this amino acid may be widespread. This could be due to the ability of tyrosine to form hydrogen bonds, or more importantly to interact with antigenic groups through the formation of hydrophobic bonds (50).

Another problem of a general nature is that of the relative contributions of the H and L chains to the antibody combining site. Although isolated L chains have never been found to possess activity (43) various authors have found that H chain preparations retained substantial binding activity (23, 52). In many cases both chains were found to be involved in the active site. Thus amino acids of both H and L chains were found to be contiguous to the combining site of antibodies directed against p-azobenzene arsonate and p-azotrimethylammonium ions by the affinity labelling technique* (49). Similar results implicating a joint contribution of both chains were also obtained with antibodies directed against the p-azo benzoate group (53) and against the f_1 bacteriophage (54). Although the data available at this time do not allow any firm generalities to be drawn, present opinion favours the view that a joint interaction of H and L chains stabilizes the active site (43). However, it is also possible that there is no general answer to this question, as further work may show that the importance of either H or L chains in a given system will be dictated by the special properties of the system itself.

The combination of antibody H or L chains with non-specific L or H chains, respectively, has beennoted in many cases (eg. ref 53), to lead to the formation of an adduct which is physically indistinguishable from either recombinants of specific antibody H and L chains or from native half I_gG molecules. The specificity of the interaction between these two chains has been shown to exist on two main levels. Thus, at the first level, recombination of antibody H and L chains in the presence of an excess of non-specific chains was found to give a

* In this technique amino acids in or near the antibody combining site are labelled by reaction of the antibody with a homologous hapten containing a reactive chemical group eg. p-(arsonic acid) benzene diazonium fluoroborate.

higher yield of binding sites than was predicted on the basis of combination being a random process (55). If peptide chains from antibodies produced to the same antigen in different animals, for example in two rabbits, are mixed, an even higher level of specificity is observed. Thus recombination between the chains of antibodies from different rabbits was observed to occur, however, active binding sites were generated only when the H and L chains were those of molecules produced by the same rabbit (56).

The Heterogeneity of Antibodies

As was shown in Table I the immunoglobulins in the serum of a given animal can be divided into three major protein classes, which are differentiated on the basis of the physical and antigenic properties of the different molecules. The antigenic differences of these classes have all been found to be due to determinants which are located on the heavy chains of the molecules (43). However there are greater antigenic complexities to immunoglobulins than those considered by their division into I_gA, I_gG, and I_gM classes. Thus the I_gG molecules of certain species, for example equine (57) and human, (58) can be divided into further subclasses on the basis of antigenic differences. Human sera have also been found to contain a fourth and fifth class of immunoglobulins, called I_gD and I_gE (59, 60). Another aspect of heterogeneity, as demonstrated by antigenic differences, is the phenomenom of allotypy. This refers to the antigenic determinants of immunoglobulins which differ among "normal" individuals of the same species. The location of the allotypic determinants on the various peptide chains and their correlation with genetic

differences are problems which have only recently been attempted (43); however, as they have no direct bearing on the work of this thesis they will not be discussed here.

Other differences besides those referred to above also exist between the different immunoglobulin classes. For example the amount of carbohydrate in I_gG immunoglobulins is usually about 2 or 3 percent, while I_gA and I_gM contain about 10 percent(43).Because of the different carbohydrate content of the different classes as well as the fact that the carbohydrate has been found to reside exclusively on the heavy chains of immunoglobulins; it has been suggested that carbohydrate may be an important antigenic determinant in immunoglobulins (43). However, it has been shown that the antigenic determinants of rabbit I_gG heavy chains were in pepsin fragments that did not contain any carbohydrate (51), which would appear to rule out its having any importance as an antigenic determinant. On the other hand, as the antibodies to the rabbit I_gG heavy chains were elicited in goats in this study (51), it is possible that carbohydrate functions as an allotypic determinant, since heteroimmune sera are generally not sensitive enough to detect these types of antigenic differences (43).

Another physical property which has provided a clear indication of immunoglobulin heterogeneity is that of electrophoretic mobility; electrophoresis in agar, starch and other gels has been widely used as an analytical tool to demonstrate this. Correlations have been found in some cases between the mobility of a parent immunoglobulin and its subunits e.g. the mobilities of F_{ab} fragments of rabbit I_gG paralled that of their parent molecules (21, 61). However, the most

interesting aspect of heterogeneity, as seen by electrophoretic criteria, is the banding of the light chains of the immunoglobulins of several species into from 6 to 10 bands when run in urea starch gels. For example, light chains of guinea pig anti-hapten antibodies form discrete bands in these gels under acid conditions, while the normal chains form a single diffuse zone (62, 63). Although the basic reasons for this phenomenon are not understood, it has been suggested to be related to the existence of populations of antibodies that may differ in structure and in affinity for the hapten (43).

The above discussion has been mainly concerned with the heterogeneity of the entire immunoglobulin population of an animal. Although antibodies reflect these types of heterogeneity, it has been found that in some cases specific antibodies to one determinant are much more homogeneous than the "normal" immunoglobulins of the animal from which they are obtained (43). However, studies of the thermodynamics of antibody-hapten interactions have indicated that in just about all systems the affinity of the antibodies for a homologous hapten are distributed over a wide range (50). For example, antibodies to the 2, 4-dinitrophenyl determinant group with binding constants differing by as much as 10^4 have been separated from the serum of an individual rabbit by fractional precipitation with increasing concentration of antigen (64).

Antibody heterogeneity is also exhibited in another way, as seen in the variation of the capacities of different segments of an antibody population to bind haptens of different sizes.

This has been shown, for example, by elution with haptens of different structure, of antibodies to the p-azobenzoate group from an

immunosorbent (65); some of the antibodies obtained could accommodate haptens with ortho substituents, while others could not. Yet another manifestation of antibody heterogeneity is the change in affinity of antibodies for their homologous haptens as a function of time after immunization of an animal. Thus a progressive rise in the average binding constant of anti-dinitrophenyl antibodies from 10^5 to 10^8 liters/mole was found in the period from 2 to 8 weeks after immunization (64, 67).

The reasons for antibody (and immunoglobulin) heterogeneity are not known. Various theories have been advanced to explain the phenomenon, based mainly on the postulates that (I) all antigens are heterogeneous, for example with respect to the environment of a hapten on a carrier protein, or (II) that the biological nature of the antibody response leads to the production of antibodies by different cell types. The idea that heterogeneity can be traced to the properties of the antigen appears to be contradicted by the result that a heterogeneous antibody response is obtained to 2, 4-dinitrobenzene coupled to one locus (lysine 41 of ribonuclease) of a homogeneous protein* (68). On the other hand some correlation between antibody affinity and immunoglobulin class has been found, i.e. I_gA and I_gG antibodies produced by an animal to the same hapten had binding constants that differed by 10^2 (69, 70). Such a result supports the thought that the basis of heterogeneity is a biological one. However, as the nature of the mechanism of antibody formation has not been elucidated an unequivocal answer to this problem cannot be given as yet.

* The possibility does arise however that this is due to (I) antibodies being directed to different portions of the hapten and carrier protein or (II) the possibility that in solution the hapten-protein conjugate can exist in antigenically different configurations.

The Reactions of Antibodies with their Homologous Antigens and Haptens: Polyvalent Antigen - Antibody Reactions

The combination of an antibody with an antigen "in vivo" facilitates removal of the antigen from circulation and thus is an important means of defence against harmful infectious organisms. Other effects of "in vivo" antibody-antigen combinations can be bothersome or harmful to the individual. For example, one can mention the symptoms of hay fever allergy and the sometimes fatal results of anaphylactic shock. However, the "biological" aspects of these reactions are so many and varied that they are beyond the scope of this introduction and therefore will not be dealt with further.

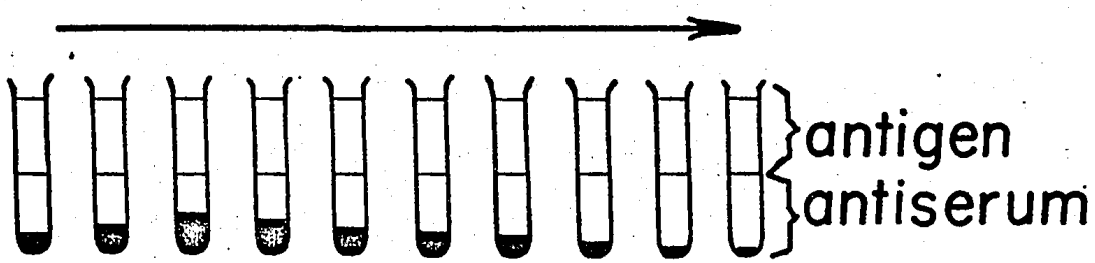
The most common "in vitro" manifestation of antigen-antibody reactions is the formation of a flocculent antigen-antibody precipitate. If the amounts of precipitate, formed by the combination of a constant amount of antibody with constant volumes of antigen solutions of increasing concentration, are plotted as a function of the amount of antigen added, the result obtained in most systems is represented by the typical graph of Fig. 2. This curve is known as a "precipitin curve" and was explained by Pauling in his framework theory on the basis of the assumption that both antigens and antibodies were polyvalent (71). Precipitation was postulated to occur as a result of the formation of a highly cross-linked insoluble network of antigen and antibody molecules. At the maximum of the precipitin curve all valencies of both antigen and antibody would be occupied and both quantitatively precipitated. An excess of either component causes the gradual destruction of the cross-

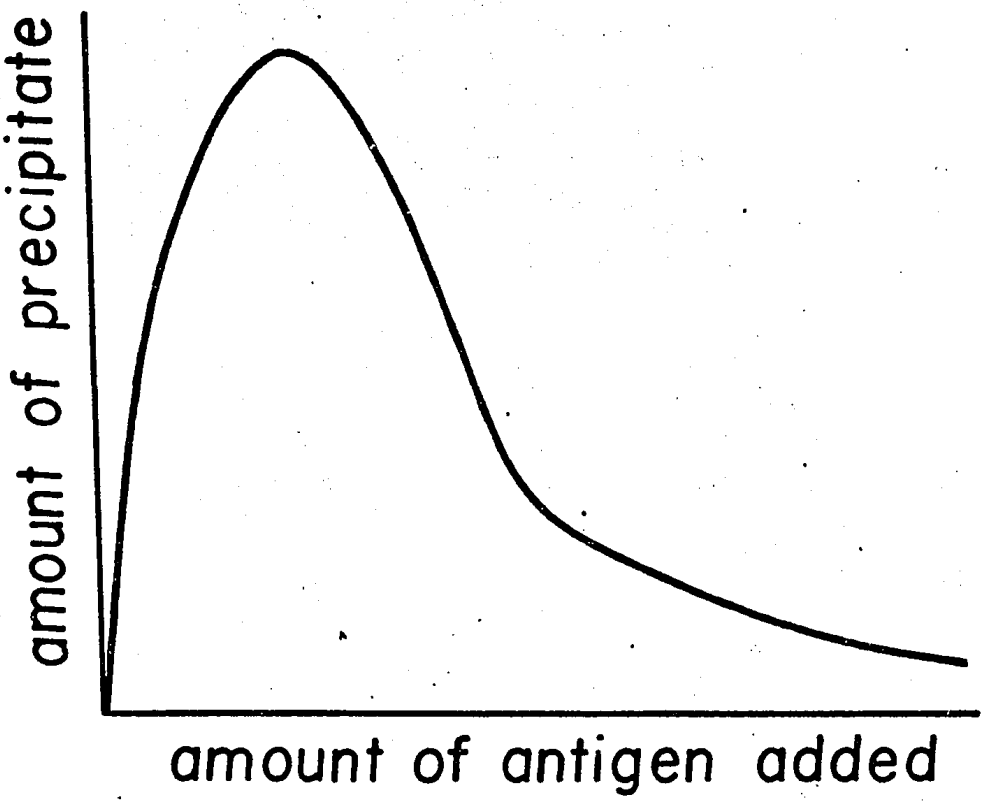
Figure 2

The precipitin curve

TYPICAL PRECIPITIN CURVE

Increasing amounts of antigen added to constant amount of antiserum





linked structure with the result that smaller and thus more soluble complexes would be formed, leading to a decrease in the amount of precipitate observed.

The premises of this theory have been fully confirmed; thus most antigens have been shown to be polyvalent, for example ovalbumin and thyroglobulin have 5 and 40 antigenic sites respectively (72) and all precipitating antibodies (except those of the I_gM class, e.g. to the azobenzene arsonate determinant are hexavalent (73)) have been found to be divalent.

Goldberg (74) has developed a mathematical description of the course of an antigen-antibody reaction as seen in the precipitin curve, based on the assumptions that a) no cyclical complexes are formed and b) that all antibody and antigen sites are equivalent. This theory centers about an expression describing the most probably distribution of antibody and antigen molecules among aggregates of different sizes. Although a detailed description of the precipitin curve was not achieved, quite accurate predictions of the ratios of antigen to antibody at which precipitation occurs were obtained.

In accordance with the above description of the antigen-antibody reaction it would be expected that the combination of either univalent antigen (or hapten), or univalent antibody with whole antibody or antigen, respectively, would not result in the formation of a precipitate. Indeed, the addition of sufficient univalent antigen or antibody to a homologous precipitating system causes the dissolution of any precipitate formed (75).

Moreover, Pauling et al (76) have developed a quantitative theory for hapten inhibition that has been used in conjunction with experiments employing haptens of varied structure to obtain information of the

molecular complementariness of antibody combining sites and to obtain values for the relative affinities of different haptens for the same antibody.

Methods of Study

A technique that is widely used to determine the minimum number of different precipitating antigen-antibody systems in a mixture is that of gel diffusion (77, 78). In this technique the antibody and antigen are placed in wells in a gel at appropriate distances from one another and then allowed to diffuse towards one another. At a region between the two where the relative concentrations of the reactants are optimal a precipitate results, corresponding to the maximum in the precipitin curve. If more than one antigen-antibody system is present, then if this optimal concentration region occurs at different locations in the gel for the different systems, more than one band will be seen. The sensitivity of the method can be increased by tagging antibody and/or antigen with radioactive tracers, while the method's ability to discriminate between the different antigenic components of a mixture has been heightened by first subjecting the antigen to electrophoresis in the gel in which diffusion will be performed (79).

Hapten-Antibody Reactions

The most unequivocal method for the determination of binding parameters of hapten-antibody reactions is equilibrium dialysis (80, 81). In this technique the hapten and antibody solutions are placed separately into two compartments of a cell, separated by a membrane which is impermeable to protein but which allows free passage of hapten. After

equilibrium is reached (the cell is usually rocked overnight) the amount of hapten bound by the antibody can be ascertained from the decrease in its concentration in the protein free compartment, after any corrections for non-specific binding (to normal proteins in the mixture or to the membrane or glass walls) have been made. If a hapten undergoes a spectral change on binding to antibody, then the extent of its combination can be measured using a method originally developed for the study of the interactions between proteins and small molecules (82, 83). If ϵ_f and ϵ_b are the extinction coefficients of the free and bound forms of the hapten respectively, and if ϵ_{app} is the overall apparent extinction coefficient for any hapten-antibody solution (all at the same wavelength), then the fraction of hapten bound to antibody, β, is given by the relation

$$\beta = \frac{\epsilon_{app} - \epsilon_f}{\epsilon_b - \epsilon_f} \qquad [1]$$

The interpretation of results obtained by such a technique may be affected by the possibility that ϵ_b is affected by antibody heterogeneity. This is discussed at length in Chapter III, part A.

In certain antibody-hapten systems e.g. using antibodies to the 2, 4-dinitrophenyl. determinant group, binding of the hapten quenches the fluorescence of the antibody. Fluorescence quenching can occur when the absorption spectrum of the bound hapten overlaps sufficiently with the emmission spectrum of the protein, provided that the orientation of the bound hapten on the protein allows an efficient transfer of radiation. A method for the determination of the extent of antibody-hapten binding has been developed that is based on this phenomenom (84). However the

results obtained by this technique may not be completely correct as it has been pointed out that in some preparations the efficiency of quenching varied as a function of the amount of hapten bound (85).

It is worthwhile at this point to mention briefly two methods which also may prove of value in studying these reactions, both from the point of view of providing binding data and of probing the antibody combining site. Thus electron spin resonance spectral changes have been reported to occur in a nitroxide containing hapten upon binding to its antibody and these changes have been used in a preliminary fashion to determine the extent of binding in this system (86). Similarly, in systems containing fluorescent haptens, the polarization of the hapten's fluorescence that might occur upon binding to antibody could be used to study these interactions. This method has been applied extensively to the study of protein small molecule interactions(163) but only one study involving antibodies, using anti ovalbumin antibodies and fluorescein labelled ovalbumin (87), has been reported to date.

Treatment of Binding Data

A detailed presentation of the various mathematical relationships useful in the analysis of binding data obtained in the study of interactions between proteins and small molecules has been given by Klotz and Karush (88, 89). Assuming that a protein has n independent binding sites with the same intrinsic affinity for a small molecule, the following relationship was derived on the basis of the law of Mass Action

$$\frac{1}{\bar{\nu}} = \frac{1}{nKc} + \frac{1}{n} \qquad [2]$$

where K is the equilibrium constant for the association reaction, $\bar{\nu}$ is

equal to the number of moles of small molecules bound per mole of protein, c is the concentration of unbound small molecules and n is the number of binding sites per protein molecule. However, in contrast to the simple linear result that would be expected from a plot of $\frac{1}{r}$ vs $\frac{1}{c}$, practically all hapten antibody systems studied have been found to be characterized by curved plots. This result was postulated by Pauling et al (90) to be due to a non-constancy of K values reflecting heterogeneity of antibody affinities within a given population of antibody molecules. This interpretation has been put into an easily used form by Nisonoff and Pressman (91) with the aid of the expression:

$$\frac{1}{b} = 1/(K_o c)^{\alpha}[Ab] + 1/[Ab] \qquad [3]$$

where c is as defined previously, b is the concentration of bound hapten, [Ab] is the concentration of antibody binding sites, K_o is the average binding constant of the whole antibody population and α is the index of heterogeneity.* The value of K_o can be obtained from a $\frac{1}{b}$ vs $\frac{1}{c}$ plot as K_o equals $\frac{1}{c}$ when half the binding sites are occupied. A value for the heterogeneity index can be calculated by plotting experimental values of $\frac{1}{b}$ vs $\frac{1}{(c)^{\alpha}}$ for various values of α, the correct value of α will produce a straight line plot.

For the determination of thermodynamic parameters of antibody-hapten reactions the following standard relations are used:

$$\Delta F^{\circ} = -RT \ln K^{\circ}$$

$$\frac{d \ln K}{dT} = \frac{\Delta H^{\circ}}{RT^2}$$

$$\Delta F^{\circ} = \Delta H^{\circ} - T\Delta S$$

where K is the equilibrium constant for the reaction being observed, R, T are the gas constant and absolute temperature, and ΔH, ΔS, and ΔF are the enthalpy, entropy, and free energy changes occurring.

*This expression was originally derived by Sips (92) to describe the absorption of a gas on a solid surface.

As the equilibrium constants determined for hapten-antibody interactions, in all systems studied, are average values, this means, therefore, that the values derived for the entropy, enthalpy, and free energy of the reactions occurring are also average parameters.

Studies of Antibody Hapten Interactions

An early example of the difference between the affinity of structurally isomeric haptens for the same antibody was obtained by Landsteiner (93) who observed that antibodies in immune sera specific for the meta-amino benzene sulfonic acid group combined to almost no extent with the ortho and para isomers of this hapten. This approach has been used in the study of many other hapten-antibody systems. For example, the importance of steric complementarity between antibody site and hapten can be seen in the extensive cross reaction of the sterically related benzene arsonate and benzene phosphonate haptens with antibodies formed against the p-azo phenylarsonate group, and in the lack of reaction between these antibodies and the similarly charged but sterically different benzoate and sulfanilate ions (94). A more subtle steric effect can be seen in the reactions of antibodies specific for pyridine. The combining site of these antibodies must accommodate the water molecules of hydration of the nitrogen atom; thus extensive cross reactions with haptens containing groups in the carbon - 4 position,such as iodobenzene; is seen (95). At another level of specificity it has been shown that antibodies can discriminate between optical isomers, as evidenced by the lack of reaction of antibody specific for the D-phenyl-benzoylamino-acetate hapten with the corresponding L-isomer (96).

The importance of steric effects in hapten-antibody reactions is a reflection of the fact that the forces involved are weak, short range forces and therefore require the closest possible approach of interacting groups for binding to occur. These forces have been postulated to be due to electrostatic interactions between charged groups, dipole-dipole interactions, Van der Waal's interactions and the formation of hydrogen and hydrophobic bonds (81 - 84) (50, 97 - 99). The importance of Van der Waal's forces can be seen in studies involving haptens containing benzene rings. For example, from a comparison of the reactivity of benzene arsonic acid and of methyl arsonic acid, which lacks the polarizable character of the benzene ring, with antibodies specific for the benzene arsonate group, it is evident that the absence of such London dispersion forces can lead to almost complete reduction of binding,* (98, p. 245). The importance of charge in antibody-hapten binding has already been implied with the mention of the finding of charges opposite to that of the determinant in the sites of antibodies formed against charged determinants. Other more direct examples of this can be seen, for example, in (i) the requirement of a doubly charged hapten for reaction with antibodies formed against a doubly charged antigenic group, e.g., O-nitro benzoic acid, which is sterically similar but only has one charged group will not interact with the antibodies directed to 4-azo-orthophthalic acid (100), and (ii) the absence of interaction between p-nitroaniline and antibodies to the p-azobenzoate group, even though this hapten is similar in size and shape to the latter molecule (83, p. 245). The

*It is likely that the difference in reactivity of these systems is also due to the difference in the extent of hydrophobic bonding in the two hapten-antibody systems.

difference in free energy of combination between the two haptens in the latter example is over 5 Kcal/mole. Assuming that these two haptens have identical shapes and sizes, calculations on the basis of Schwartzenbach's (101) evaluation of the effect of distance between charges in water on free energies of interaction, showed that this difference could arise from a charge separation of 4.5 Å (98, p. 249). This would be about the distance of closest approach of a carboxylate and ammonium group in water.

Thermodynamic Studies

The results of thermodynamic studies of some typical antibody-antigen and antibody-hapten systems are listed in Table II. As can be seen the free energy changes, except in the case of the dinitrophenyl system, fall in the range between -5 to -8 Kcal per mole, indicating that although the forces involved in these reactions are known to be highly specific they are not necessarily very strong.

A consideration of the fact that association reactions result in losses of rotational and translational degrees of freedom would lead one to expect, at first sight, that antigen-antibody reactions would be characterized by negative entropy changes. Experimental results, however, have shown that this is not so; indeed, most of the systems studied involved positive entropy changes which represent an important contribution to the free energy change of the overall reaction. The reason for this has been postulated to lie in the formation of a "hydrophobic bond" when antibody and antigen or hapten associate, implying that the forces involved in the binding process are similar to those

Table II

Thermodynamic Constants for Antibody-Antigen Reactions

System	ΔF° (kcal/mole)	ΔH° (kcal/mole)	ΔS° (e.u.)	Reference
Benzoic acid: anti-p-azobenzoate antibodies	-6.1			(102)
ε-N-dinitrophenyllysine: anti-DNP-antibodies	-6.8	-1.6	17	(103)
ε-N-dinitrophenyllysine: anti-DNP-antibodies	-11.3	-8.6	9	(84)
p-(p-dimethylaminobenzeneazo)-phenyl β-lactoside(Lac-dye): anti-Lac-dye antibodies	-7.09	-9.7	-8.8	(104)
D-phenyl-(p-(p-dimethylaminobenzeneazo)-benzoyl-amino)-acetate (D-I_p-dye): anti D-I_p-dye antibodies	-7.25	-7.1	0.3	(96)
p-(tyrosineazo)-benzene sulphonic acid: anti-p-azobenzene sulphonate antibodies	-8.97	-8.39	2	(105)
Terephthalanilide-p,p'-diarsonic acid: anti-benzene arsonate antibodies	-7.4	0.8 ± 26	22 ± 9	(106)
Bovine serum albumin (BSA): anti-BSA antibodies	-5.5 ± 0.2	0 ± 2	20 ± 8	(107)
Ovalbumin: antiovalbumin antibodies	-5.6 ± 0.2	0 ± 2	20 ± 8	(33)
Multivalent BSA-azobenzene arsonic acid: anti-benzene arsonate antibodies	-4.8 ± 0.2	0 ± 1	18 ± 4	(108)

responsible for the immiscibility of simple hydrocarbons in water (109). Thus the dissolution of a nonpolar molecule in water is thought to cause a perturbation of the bulk water structure so that water molecules about non-polar residues are arranged in a quasi-crystalline "iceberg" structure (100). If two non-polar residues (e.g. antibody combining site and hapten) can interact closely the water of solvation (iceberg water) can be released, resulting in a positive entropy change. This type of interaction has been postulated to be an important factor in stabilizing the native conformation of a protein in water (109, 111). In the same way the interaction of an intrinsically insoluble haptenic group such as the 2, 4-dinitrophenyl residue, or other haptens containing aromatic determinants, with an antibody site could well be extensively stabilized by the release of solvent associated with the formation of such hydrophobic bonds (99, 109, 112).

A comparison of the results listed in Table II for the reactions of antibodies with protein antigens and haptens, respectively, show that both types of systems are generally characterized by very similar free energy changes. However, the enthalpy changes of the protein antigen systems are very small, whereas, with the exception of the dinitrophenyl and arsonic acid systems the enthalpy changes of hapten-antibody reactions are quite large and make a relatively more important contribution to the free energy change of the reaction. Unfortunately, however, in view of the exceptions noted and the problems of antibody heterogeneity, it has not been possible to correlate these differences in thermodynamic behavior with the mechanisms involved in the reactions of these two different types of systems.

Kinetic Studies

In the study of the kinetics of polyvalent antigen-antibody reactions it is imperative to limit rate measurements to the region of antigen excess, i.e. to the formation of soluble complexes, so as to avoid complications due to the formation of antibody-antigen precipitates. A few kinetic studies based on this approach have been reported using, for example, light scattering to distinguish between antigen, antibody and their adducts on the basis of size (113, 114, 115). However, unequivocal interpretations of the kinetic results of polyvalent antigen-antibody systems are difficult to make (116). Thus the fact that many different and complex reactions occur simultaneously may obscure the measurement of the primary combination of antigen and antibody. Also the complexes formed may undergo re-equilibration reactions among themselves, while repulsion between large protein antigens may lead to the binding of successive antigens to the same antibody occuring at different rates.

These difficulties have been overcome by the use of monovalent hapten-antibody systems for kinetic experiments. The association reactions in the systems studied have been found to be very rapid, with rate constants approaching those of diffusion controlled processes (117). For example, the forward rate constant for the reaction of antibodies to insulin and this protein, which has only one antigenic site per molecule, has been found to be about 10^9 M^{-1} sec^{-1} (118). This reaction could be studied by a classical kinetic approach by employing very low, i.e. 10^{-9} M, concentrations of I^{131} tagged insulin. Values of the same range, i.e. 2×10^7 to 2×10^8 M^{-1} sec^{-1} have been found for the forward rate constants in systems consisting of antibodies to the 2, 4-dinitrophenyl (119)

nitrophenyl (120) and phenylarsonate (121) determinant groups and homologous haptens, while the reverse rate denstants varied from 1 to about 760 sec^{-1}. Special techniques, for example stopped-flow techniques in combination with fluorescence quenching, have proven useful in these studies (119). Another method which has been applied, in this laboratory and during this investigation, to the study of these systems is the temperature-jump technique (122). This method is described in detail in Chapter III.

PART B

Bovine Serum Albumin and Its Interaction with Small Molecules

The (serum) albumin molecule is one of the most thoroughly studied of the animal proteins. Although it is not known to possess any enzymic or hormonal activity it has been shown to have the unusual ability to combine strongly with a variety of smaller molecules ranging from halide ions to detergents. There are at least three reasons (123) for the study of the interactions of this protein with small molecules, i.e., a) understanding of physical properties such as solubility, titration behavior, etc. requires a knowledge of the degree of binding of ions of all types in a given situation, b) such studies may help in elucidation of protein structure, and c) the binding of small molecules may be related to the protein's biological functions. It has also been suggested that another important reason for the attention paid to this protein is that it is readily available in large quantities and in reasonable purity.

Physical Properties

Albumin is most easily differentiated from other serum proteins

by its high electrophoretic mobility. Thus at pH 8.6 $\mu = 0.1$ albumin has the highest net charge of the plasma proteins.* It has a molecular weight of 66,000 (125) and is generally considered to consist of a single polypeptide chain (126).

The elucidation of the shape and size of the plasma albumin molecule has been found to be complicated by the variation in these properties with changes in pH or ionic strength. Observations based on viscosity, optical rotatory dispersion, and solvent perturbation studies have indicated that in the pH range from 4.2 to 8 the molecule exists as a fairly compact and rigid entity (127). However at pH values outside this range a marked variation has been found. Thus the expansion of the molecule at low pH, originally postulated by Tanford on the basis of titration data (128), has been confirmed by other investigators on the basis of viscosity (129, 130) and depolarization of fluorescence measurements (131). Also, recent electron microscopic studies (132) have shown that the bovine serum albumin (BSA) molecule undergoes a change from a globular particle (major and minor axes of about 60 and 45 Å) at neutral pH, to a threadlike structure approximately 250 Å long and 21 Å in diameter at pH 1.9.

BSA also exhibits unusual electrophoretic properties at low pH. This behavior has been extensively studied by Foster and coworkers, who postulated that the heterogeneity of BSA preparations as seen electrophoretically, is due to the existence of a transition (called N-F for normal to fast) in the molecule's structure associated with the uptake

* Pre-albumin, only occasionally found in sera, has a higher net charge than albumin (124).

of hydrogen ions (133). Two aspects of the N-F transition deserve further mention. Firstly, although theoretical calculations predicted that the transition should be 12th order in hydrogen ion, experimentally it seemed to be only third order. Secondly, although N and F forms could be separated electrophoretically it was found that the equilibrium between the two forms was reached in times much shorter than that of the electrophoretic experiment.

These two seemingly paradoxical results were explained by Foster's proposal that i) the BSA molecule was composed of four similar (connected) subunits each undergoing an independent transition which was third order in H^+ ion (134) and ii) that the molecules of a BSA preparation were not characterized by having identical N-F transition pKs, but rather that a distribution of these pK values existed.

Recently, physical proof for part of this first postulate has been obtained by electron scattering experiments (135), which were consistent with a subunit structure at pH 3.6 (albeit of three units), and by enzymic fragmentation studies (136) where an observed rapid and then slow release of protein fragments was found to be best explained by postulating that the molecule consisted of 3 or 4 globular segments linked together by more flexible portions of the peptide chain. Support for the second postulate has been obtained by the demonstration that subfractions of an albumin sample undergo N-F transitions at different pH's (137).

Binding of Small Cations and Anions

Bovine serum albumin binds both univalent and divalent cations,

the latter much more strongly due to increased Coulombic attraction. Thus sodium is hardly bound at all (138) while calcium is bound to an appreciable extent (139). In the case of the transition metals, for example, iron, copper, chromium and zinc, it has been found that the ability of these ions to chelate with various electron donor groups on the protein through their unoccupied orbitals leads to stronger binding than that expected from Coulombic interactions and, indeed in some cases, to reactions of considerable specificity. For example the reaction of the H_g^{++} ion with SH groups of mercaptalbumin (albumin containing a free SH group per molecule) is quite strong (the equilibrium constant for this reaction being about 10^7) (140) and is used as a means of quantitatively separating merceptalbumin from an albumin preparation (141) through the formation of a mercury-albumin dimer. Similarly, zinc and cadmium ions have also been shown to be strongly bound (142). The results of such studies have been interpreted to mean that these ions are bound to both imidazole groups and neighboring peptide nitrogen or oxygen atoms.

The binding of small anions would be expected to proceed to a similar degree as that of cations of equal change. However, in all but strongly alkaline solutions it is found that anion binding is so great that the binding of cations can effectively be ignored (123, p. 192). The relative affinities of BSA for the most common anions found in protein studies are in the following order: bicarbonate $\angle$ acetate $\angle$ chloride $\angle$ bromide $\angle$ citrate $\angle$ nitrate $\angle$ iodide = thiocyanate (143, 144).

The effect of bound anions on the properties of BSA is quite important. For example the difference in the titration curves of BSA at low pH in the presence of chloride and thiocyanate ions is directly attributable to the stronger binding of the thiocyanate ion causing a reduction in electrostatic free energy (at a given pH) through reduction of the proteins net positive charge. The binding of anions has also been found to lead to shifts in isoelectric points (145), to stabilization against tryptic hydrolysis (146), and to a change in the N-F transition (147).

Binding of Detergents

In contrast to albumin's unique affinity for small ions, the ability to bind detergents is a property shared by all proteins so far investigated (123, p. 197). Also, detergent binding has been found to have many different effects on protein properties, leading, for example, to precipitation, solubilization and protection against denaturation (148). Initial observations of protein-detergent systems led to the belief that binding was due solely to charge interaction. For example, in the binding of sodium dodecyl sulfate to horse serum albumin a 1:1 correspondence between anionic detergent molecules bound and cationic protein sites was found (149). However, considerations of the hydrophobic nature of detergents as well as more recent results, e.g. zein (127) and serum albumin (123, p. 200) have been shown to bind about 20 and 3 moles of detergent per protein cationic site, have made it clear that these reactions are most likely highly stabilized through hydrophobic bond formation.

One of the most interesting aspects of albumin-detergent interactions are their so-called "cooperative" nature. Thus, for example, in electrophoretic studies of certain detergent-albumin mixtures, e.g. sodium p-octyl benzene sulfonate and BSA (129), the first 12 moles of detergent bound per protein molecule were found to react with independent binding sites. However, as the concentration of detergent was increased complexes were observed that appeared to be formed by the binding of a large number of detergent ions in a single "cooperative" step.

Decker and Foster (150) have formulated a theoretical description of albumin-detergent interactions based on the assumption that the protein could exist in three isomeric forms A, B and C. Form A would have 12 independent binding sites while 38 and 76 detergent molecules could be bound, perhaps co-operatively, to a BSA molecule in form B or C, respectively. On the basis of this postulate they proposed two mechanisms to describe the behavior of these systems. The first was based on co-operative binding by forms B and C; the detergent-protein complexes formed were postulated to be similar to detergent micelles, stabilized in this case by the detergent-protein bonds formed. In the second mechanism cooperative binding was not invoked; the constancy of composition of the complexes of the B and C forms was explained by postulating that the intrinsic binding constants of these protein isomers for detergent were so strong (values of $K \cong 3 \times 10^6$ gave good agreement) that the formation of detergent complexes was complete when significant concentrations of forms B and C existed in solution.

As it was found that the experimental data available could be described by both of these very different mechanisms, the authors suggested that experiments based on different approaches were needed before these interactions could be fully understood.*

Binding of Aromatic Molecules and Azo Dyes

Observations of the binding of dyes to proteins date back approximately 60 years to Sørensen (151) who pointed out that spectral changes due to these interactions could lead to large errors in the determination of the pH of protein containing solutions by colorimetric indicators. Since this initial observation, studies have been made that have demonstrated spectral changes in many different albumin-dye systems. These changes have been found useful in obtaining binding data (152), (82,83) in interpreting binding mechanisms (153), and, in the case of a small number of dyes, for quantitatively determining the amount of albumin in human plasma (154, 155). On the whole, however, spectral studies of dye - BSA interactions have not provided the insight that original investigators hoped for. A premonition of this can be seen from Klotz's early observation that in a comparison of the binding of dyes such as orange I and II to BSA, no correlation between the strength of binding and spectral change was found (156).

A comparison of the reactions of azo dyes with BSA and with specific antibodies is instructive. For example, antibodies specific for the azophenyl-arsonate group bind the hapten p-N, N-dimethylaminophenyl-azo-p^{1}-benzene arsonic acid strongly (ΔF° = -7.7 Kcal/mole), while the

*Another complicating factor in these systems is the possibility that BSA microheterogeneity (mentioned previously with respect to resolution of N and F forms) may affect observations of detergent binding.

benzene sulfonic acid analogue of this hapten is hardly bound at all (148). In contrast BSA binds the benzoic, benzene sulfonic and benzene arsonic acid analogues of this dye with approximately the same affinity (149). However, although the specificity observed in these systems does not compare with that seen in hapten-antibody reactions, structural isomers can exhibit striking differences in behavior. Thus in a study of the o-, m-, and p-carboxyl isomers of p-[N, N-dimethylaminophenyl-azo] benzoic acids, Klotz (157) found that the binding of the ortho isomer was very different from that of the meta and para forms. An explanation postulated for this was that binding to the protein involved simultaneous interaction of the dimethyl amino and carboxyl groups of the dye with tyrosine and a positively charged side chain of the protein, respectively. If these side chains were about 12 Å apart then, as molecular models showed, only the m- and p- isomers could interact in this way, the distance between groups in the o-form being too small. The fact that the cinnamic acid derivative of the p-isomer, in which the dimethyl amino to carboxyl group distance was 14 Å and therefore, too great for interaction, behaved the same way as the o-isomer further substantiated this idea.

It is interesting to note that, in general, the reactions of BSA with dyes and other small molecules are characterized by free energy changes which are similar in magnitude to those found for antibody-hapten systems (158, 159). This result seems paradoxical in view of the relative non-specificity of the BSA reaction. It has been suggested (160) that the explanation for this lies in this protein having great configurational adaptability, such that, when binding occurs, optimal interactions between it and many different small molecules are possible.

In recent years, new methods have been applied to the study of albumin (and protein) - ligand interactions. Thus, the use of nuclear magnetic resonance (NMR) for example, promises to provide new insight into such binding reactions, for by this technique the loci of interaction may be pinpointed on the basis of a change in the NMR spectrum of aligand (161). This has proven possible in a few systems so far; for example peak broadening in the penicillin-BSA system (162) was interpreted as meaning that the principal site of interaction was the phenyl ring of this molecule and similarly, studies of the binding of various sulfonamide derivatives (163) showed that the important point of their attachment to BSA was through the parent p-amino benzene sulfonamide residue.

Polarization of fluorescence, developed by Weber and Laurence (164) is another important technique. In this method binding of a (small) fluorescent molecule can be detected because the decrease in its speed of rotation when fixed to a protein (relative to that in solution) leads to a polarization of its fluorescence. Studies based on this phenomenon have allowed the calculation of binding parameters and the development of certain generalizations concerning the nature of interactions between proteins and small molecules. Thus Laurence (165) was able to conclude from parallel studies in cetyltrimethyl ammonium bromide and dioxane (the former a micelle-forming detergent and the latter a solvent of low dielectric constant) that the binding of 1-naphthylamine 8-sulfonic acid to BSA was at a site of low dielectric constant. It is also worth mentioning that the importance of hydrophobic bonds in BSA-small molecule interactions has also been demonstrated by Hansch (171) who

showed that the binding of a series of derivatives of phenol to BSA closely paralled the extent of their transfer from water to octanol.

Scope of this Study

This thesis is concerned with an investigation of the equilibrium and kinetic aspects of the reactions of two azo dyes, 1-Napthol 4-(2, 4-dinitrophenyl azo) 2-sulfonic acid and 1-Napthol 2-(2, 4-dinitrophenylazo) 3, 6-disulfonic acid, with bovine serum albumin and with rabbit antibodies specific for the 2, 4-dinitrophenyl determinant group. The two dyes were chosen mainly on the basis of their having two properties, i.e., both contained the 2, 4-dinitrophenyl group and both exhibited large spectral changes upon binding to the above proteins under suitable conditions.

One of these dyes, 1-Napthol 2-(2, 4-dinitrophenyl-azo) 3, 6-disulfonic acid had been previously found to exhibit a marked spectral change when it reacted with specific antibody (166). The second dye used was synthesized by the author in the expectation that it would also behave in a similar manner on binding to proteins.

The results of studies performed have been divided into three main sections:

1) The behavior of these azo-dyes in the presence of bovine serum albumin was investigated and the effects of pH and various inhibitors on the reaction were evaluated.
2) A comparative study was made of the binding of 1-Napthol 2-(2, 4-dinitrophenyl azo) 3, 6-disulfonic acid to anti-dinitrophenyl antibodies as determined by equilibrium dialysis and a spectrophotometric technique.
3) The kinetics of the reactions of these dye-haptens with anti-dinitrophenyl antibodies were studied, primarily with the temperature-jump relaxation technique; exploratory experiments were performed with a Durrum Gibson stopped flow apparatus.

CHAPTER II

The Interaction of 1-Napthol 4-(2, 4-Dinitrophenylazo) 2-Sulfonic Acid and Related Compounds with Bovine Serum Albumin

INTRODUCTION

Bovine serum albumin (BSA) has been found to interact with a great variety of small organic molecules. Of these interactions the most interesting are those that lead to spectral changes of the bound ligand, for the possibility exists of finding a relation between the spectral changes and the forces responsible for the binding reaction. Some azo-dye pH indicators have been found to exhibit marked visible spectral changes upon binding to BSA (167, 168, 169). The dye 1-Napthol 4-(2, 4-dinitrophenylazo) 2-sulfonic acid has also, as will be described in this and the following chapter, been observed to undergo a large spectral shift, both when binding to BSA or to rabbit antibodies specific for the 2, 4-dinitrophenyl residue. The interaction of this dye with BSA was, therefore, investigated both to obtain a comparison of the behavior of these two protein-dye systems and a clearer understanding of the mechanism of these spectral shift(s).

MATERIALS

Crystalline BSA was obtained from Armour Pharmaceuticals, Kankakee, Ill. BSA solutions were dialyzed extensively against the

appropriate solvent before use. The molecular weight of BSA was taken as 66×10^3 (170) and its extinction coefficient $E^{1\%}_{cm}$ at 279 mμ as 6.67 (150).

Sephadex G-50 Fine was obtained from Pharmacia, Upsalla, Sweden.

Buffers of ionic strength, μ, 0.02, and containing no supporting electrolyte, were used because of the decreased solubility of the dyes at higher ion concentrations. The following buffers were employed for different pH ranges:

pH range	Buffer
2 - 4	Glycine - HCl
4 - 6	Acetate
6 - 8	Phosphate
8 - 10	Borate

The structural formulae of the various azo-dyes and of some of the other related molecules used in this study are given in Fig. 3. The methods of preparation of the azo dyes used are given below.

PREPARATION AND PROPERTIES OF AZO-DYES

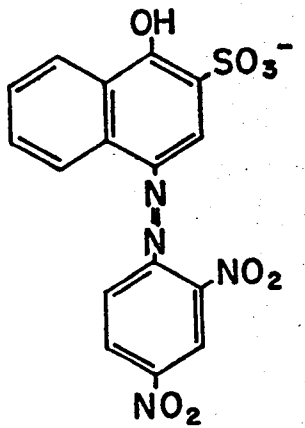
A) 1-Napthol 4-(2, 4-dinitrophenylazo) 2-Sulfonic acid [1N-2S-4DNP]

This dye was synthesized* according to the method recommended for the coupling diazonium salts to napthols (172). The diazonium salt of 2, 4-dinitroaniline was prepared by dissolving 0.366 g (2×10^{-3} mole) of this substance in 3 ml of concentrated sulfuric acid containing 0.14 g

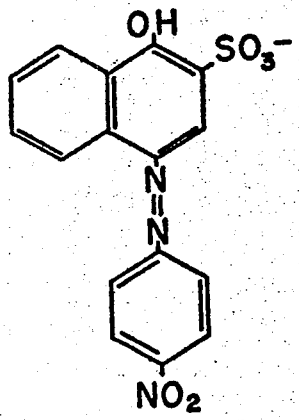
* This dye had been previously synthesized, using a similar method, by Akegami and Hujuma (173); this information was discovered after the author had prepared the dye by the procedure described.

Figure 3

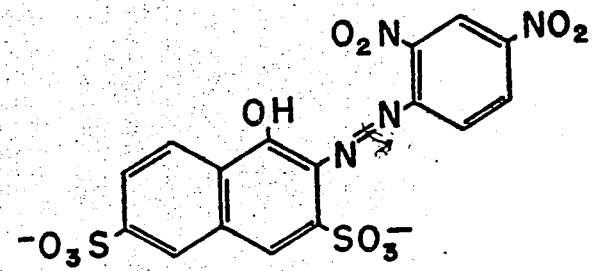
Structural formulae of azo dyes and related molecules used in this study



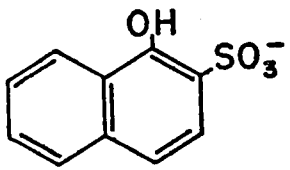
1N-2S-4DNP



1N-2S-4pNP

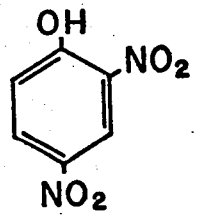


1N-3,6S-2DNP



1-Napthol-2 Sulfonic-acid

4-Nitrophenol



2,4-Dinitrophenol

sodium nitrite. The solution was then stirred at room temperature for about 1/2 an hour. When diazotization was judged to be complete; i.e. when no precipitate formed on adding drops of the solution to water (172), the solution of the diazonium salt was poured onto ice chips. The cold solution was then added slowly to a cold solution of a 5 molar excess of the 1-napthol 2-sulfonic acid, maintained at a pH of 8 to 10 during the coupling reaction by the continual dropwise addition of 3N sodium hydroxide.

The resulting orange colored dye was purified by two precipitations from basic solution with hydrochloric acid and finally by recrystallization from hot water. The identity of this compound was confirmed by analysis of the dried product by Daessle Organic Microanalysis, Montreal, Quebec, given below:

	%C	%H	%N
Calculated for $C_{16}H_{10}O_8N_4S.H_2O$	44.0_0	2.7_8	12.8_2
Found	43.9_3	2.7_5	12.7_3

B) 1-Napthol 4(4-nitrophenylazo) 2-Sulfonic Acid (1N-2S-4pNP)

This dye was prepared in a similar manner to 1N-2S-4DNP. In this case 0.222 g p-nitro aniline was diazotized with 0.110 g sodium nitrite. The cold diazonium salt solution was then added to a cold solution of a 5 molar excess of the napthol sulfonic acid and the resultant dye was purified by two recrystallizations from hot acidic ethanol-water solutions.

METHODS

A) Binding Measurements

It was found that at pH 5 the dye IN-2S-4DNP was extensively bound to dialysis membranes.* This therefore precluded the use of equilibrium dialysis techniques; however, as this dye underwent a large bathochromic spectral shift upon binding to BSA at this pH, the method developed by Klotz (83) for the calculation of dye binding could be profitably used. As mentioned in Chapter I, the fraction of dye molecules bound in such a case is given by the expression

$$\beta = \frac{\epsilon_{app} - \epsilon_f}{\epsilon_b - \epsilon_f}$$

The extinction coefficient of the bound form of the dye, ϵ_b, was calculated in a manner similar to be described in Chapter III, i.e. by the addition of dilute dye solutions to solutions containing a constant high concentration of BSA. As it was anticipated that the affinity of BSA for this dye would be lower than that of an homologous antibody, the protein solutions used for these measurements were of sufficiently high concentration (approximately 5%) to assure that all dye present would be bound.

In binding experiments the ratio of dye to protein was of course much higher than in those solutions used to determine ϵ_b. The amount of dye bound as a function of free dye present in such experiments was represented in terms of the equation of Nisonoff and Pressman (91), i.e.

$$1/b = 1/K_o c[P] + 1/[P] \quad **$$

where [P] is the concentration of protein binding sites, and b, c, and K_o

* Visking dialysis membranes, treated to remove impurities as described in Chapter III, Part A, were used.
** This equation is equivalent to equation (3) of Chapter I.

are as defined previously. Values of K_o, the average equilibrium constant, and [P] were then obtained from this plot as detailed in Chapter I.

The effect of different compounds on the spectral shift of IN-2S-4DNP produced by BSA was evaluated by adding these materials to mixtures of IN-2S-4DNP and BSA of known composition. The pH of the final solution was verified in each case with a Radiometer pH meter.

Sephadex G-50 gel columns (1.1 x 10 cm) adjusted so as to give flow rates of approximately 25 ml/hr were used for gel filtration experiments; similar techniques have been used for binding studies in other systems (174, 175).

In cases where the effect on the spectral shift of IN-2S-4DNP of an inhibiting compound was to be evaluated, the gel was equilibrated with a buffered solution of the inhibitor at the same concentration of this compound used in the dye-protein solution.

Hydrogen ion titrations of BSA-dye complexes were done by the addition of hydrochloric acid ($\sim$0.5N) to protein-dye solutions. The ratio of protein to dye concentrations in these latter experiments was kept sufficiently high, approximately 200, to ensure that all dye present was bound. A Radiometer pH meter, calibrated at pH 2, 4 and 6 with standard buffers was used, and optical densities were corrected for any small volume change due to addition of acid.

All experiments were done at 23-25°C.

All spectra were measured in a Unicam SP 500 spectrophotometer and corrected for protein or competitor absorption by the use of appropriate blanks.

RESULTS AND DISCUSSION

Properties of the Dyes

Both IN-2S-4DNP and IN-2S-4pNP have the properties of pH indicators, changing from yellow in acid to blue in basic solutions [Figs. 4, 5], their spectral shifts most likely being due to ionization of the naptholic OH group and rearrangement of the molecules to quinonoid structures. Spectrophotometric titrations of the dyes, at 600 mμ for IN-2S-4DNP and at 585 mμ for IN-2S-4pNP, in buffers of different pH (and at $\mu = 0.02$)* gave the identical value of 7.64 for the pK of the naptholic OH group [Fig. 6]. Both dyes obeyed Beer's law in the region of concentrations used in this study i.e. IN-2S-4DNP obeyed Beer's law up to a concentration of approximately 2.2×10^{-5} molar at pH 5, $\mu = 0.02$. At higher pH, even more concentrated solutions could be used because of the greater solubility of the ionized forms of these molecules. Solutions of both these dyes were stable over a period of months at the pH values used, as indicated by the constancy of the optical density values of their solutions.

For the properties of 1 Napthol 2-(2, 4-dinitrophenylazo) 3, 6-disulfonic acid, (1N-3, 6S-2DNP) please see Chapter III, Part A.

Protein Dye Interactions

The effect of various amounts of BSA on the spectrum of a 1.1×10^{-5} M solution of IN-2S-4DNP at pH 5 $\mu = 0.02$, is shown in [Fig. 7]. As can be seen, interaction with the protein markedly shifts the spectrum of the bound dye to one quite similar to that of the free ionized

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* The pK's of these dyes at $\mu = 0.1$ were found to be 7.42. The dye solutions used for experiments of this ionic strength were fairly dilute, about 5×10^{-6} M, because of the lower solubility of the dyes.

Figure 4

Spectrum of a 1N-2S-4DNP solution (4.1×10^{-5} M) at pH 3.2 (Curve #1) [Acetate buffer, $\mu = 0.02$] and pH 10 (Curve #2) [Borate buffer, $\mu = 0.02$]

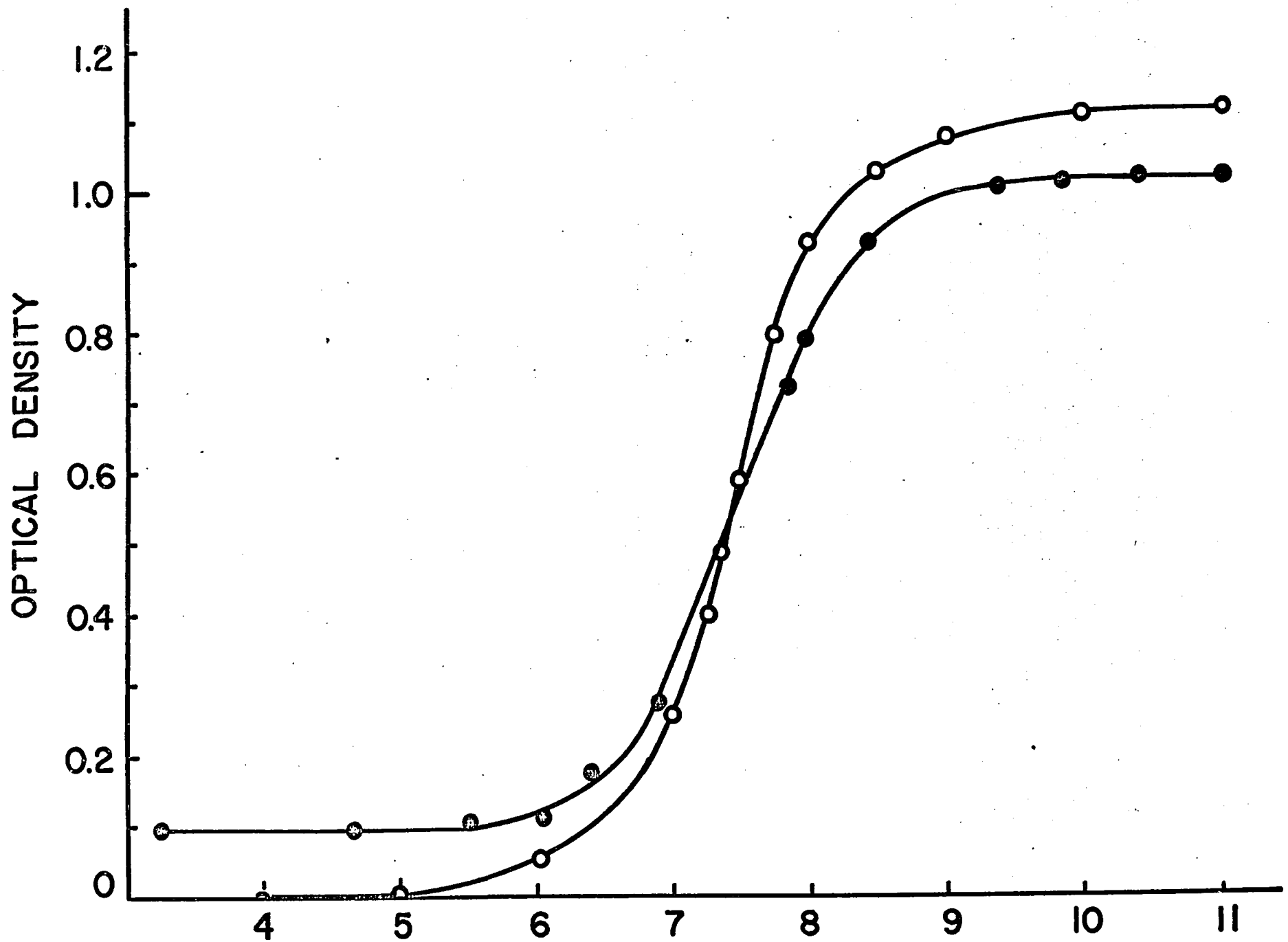
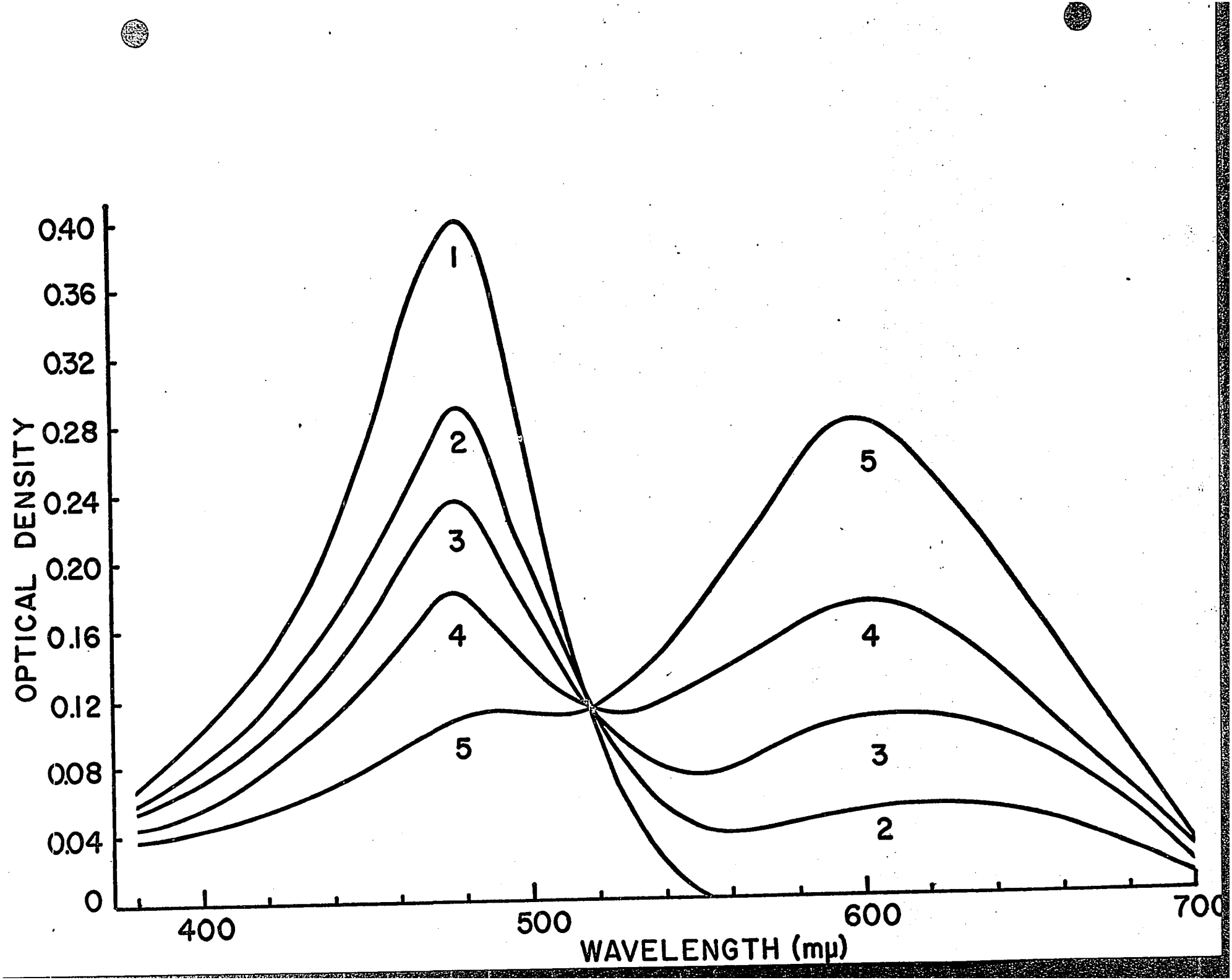

OPTICAL DENSITY
1.2
1.0
0.8
0.6
0.4
0.2
0
4
5
6
7
8
9
10
11

Figure 7

Effect of BSA on the spectrum of a 1.1×10^{-5} M solution of 1N-2S-4DNP at pH 5, μ= 0.02. Curve #1 - buffer only. Curve #2 - 0.9×10^{-6} M BSA, Curve #3 - 1.8×10^{-6} M BSA, Curve #4 - 3.9×10^{-6} M BSA, Curve #5 - 40×10^{-6} M BSA


0.40
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
OPTICAL DENSITY
1
2
3
4
5
5
4
3
2
400
500
600
700
WAVELENGTH (mμ)

form [c.f. Fig. 4], while the extent of the change produced depends on the concentration of BSA used. However, at pH 9.5, where ionization of the dye is complete, the effect of BSA on the spectrum of the dye [Fig. 8] is much less dramatic, even at a protein concentration 2.5 times higher than the most concentrated used at pH 5. At this pH only a small bathochromic shift of approximately 25 mμ, and a slight decrease in extinction coefficient, are seen.

The results of the interactions of IN-2S-4pNP and IN-3, 6S-2DNP with BSA under the same conditions which lead to such marked changes in the spectrum of IN-2S-4DNP are quite different. In the case of IN-2S-4pNP at pH 5, μ= 0.02 [Fig. 9] one observes only a decrease in extinction coefficient, while at pH 9.5, binding of the dye leads to an effect which is similar to that produced with IN-2S-4DNP at this pH (see Fig. 8 for the latter). The interaction of IN-3, 6S-2DNP with BSA at pH 5 μ= 0.02 [Fig. 10] leads to a shift similar to that of IN-2S-4DNP but of much smaller magnitude.

Determination of Binding Parameters for the Reaction of IN-2S-4DNP with GS/4

The extinction coefficients at pH 5, μ= 0.02 of the bound dye at 480 and 600 mμ, the absorption maxima of the protonated and ionized free forms of the dye, respectively, were determined by the addition of solutions of IN-2S-4DNP of different concentrations to solutions of BSA at a final concentration of the latter of 4.2×10^{-4} M [Fig. 11]. As the protein was present in considerable excess in these solutions (approximately 100 fold molar excess) it was assumed that all the dye present was bound.

Figure 8

Effect of BSA on 1N-2S-4DNP (2.2×10^{-6} M) at pH 9.5, μ= 0.02. Curve #1 - buffer only, Curve #2 - 1×10^{-4} M BSA

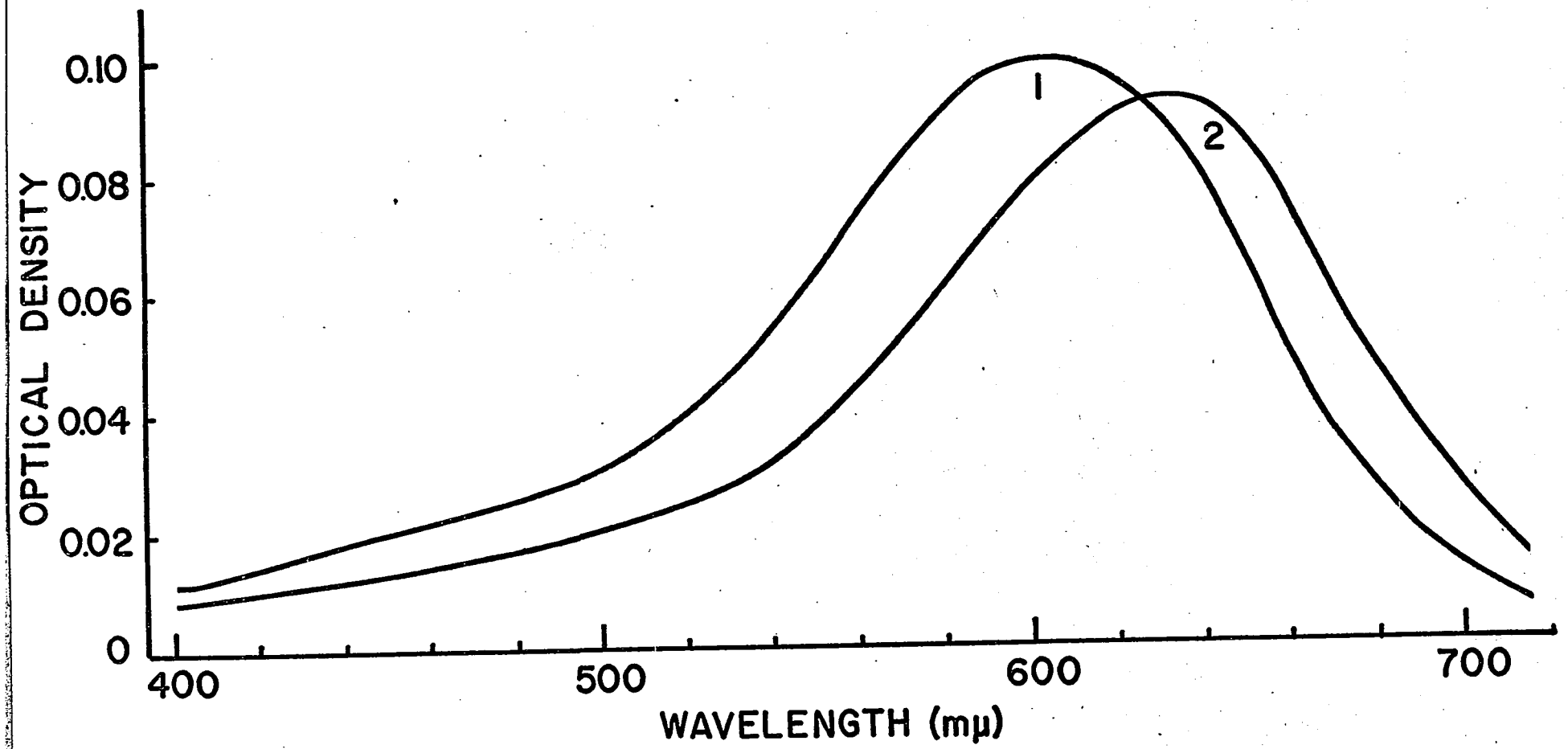

0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
400
500
600
700
WAVELENGTH (mμ)
1
2

Figure 9

Effect of BSA (3.6×10^{-6} M) on the spectrum of IN-2S-4pNP (1.1×10^{-5} M). Curve #1 - Buffer, pH 5 μ= 0.02, Curve #2 - Buffer pH 5, containing BSA, Curve #3 - Buffer pH 9.5, μ= 0.02, Curve #4 - Buffer pH 9.5 containing BSA

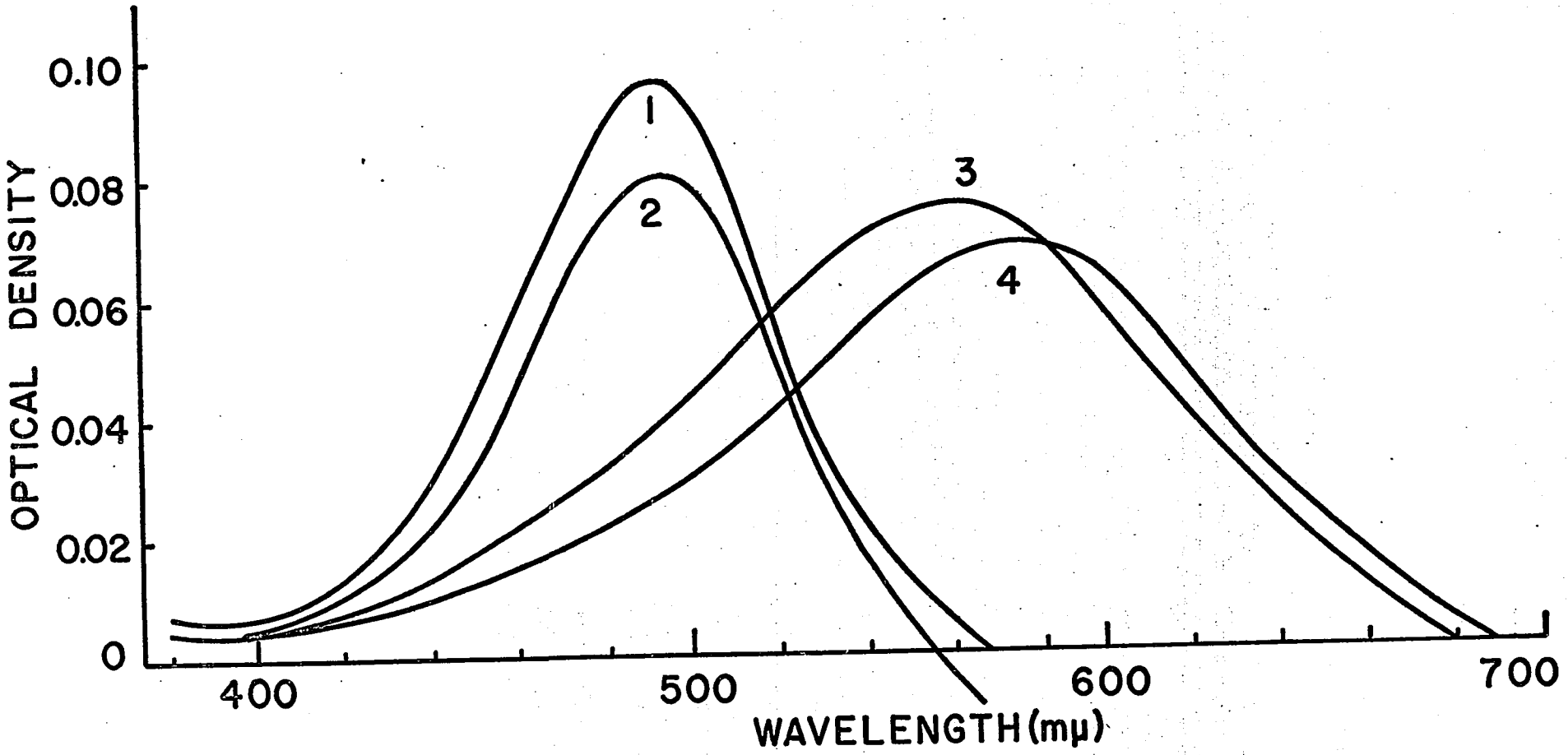

0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
1
2
3
4
400
500
600
700
WAVELENGTH (mμ)

Figure 10

Effect of BSA (3.6×10^{-6} M) on the spectrum of IN-3, 6S-2DNP (1.1×10^{-5} M) at pH 5, μ = 0.02. Curve #1 - Buffer only, Curve #2 - BSA.

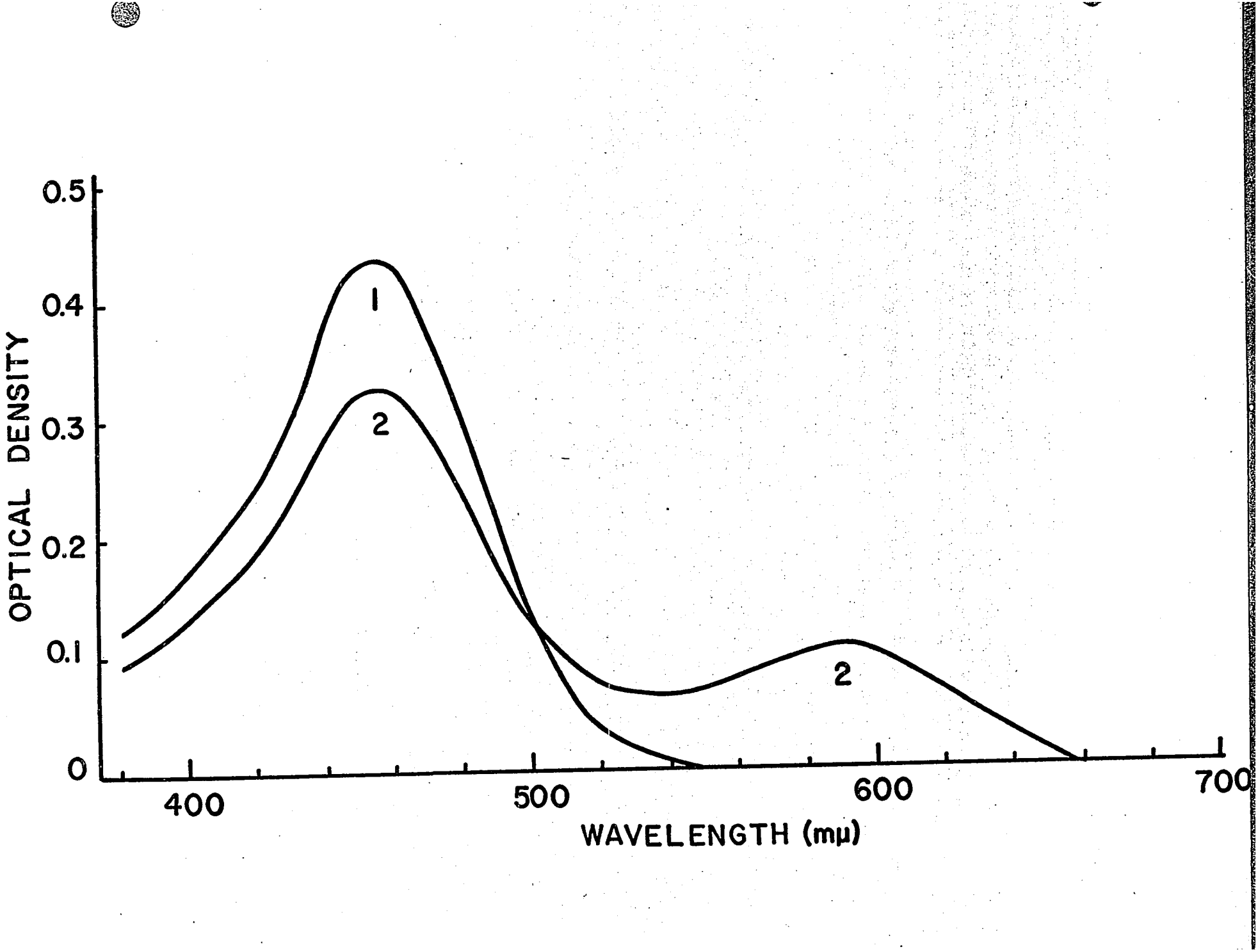
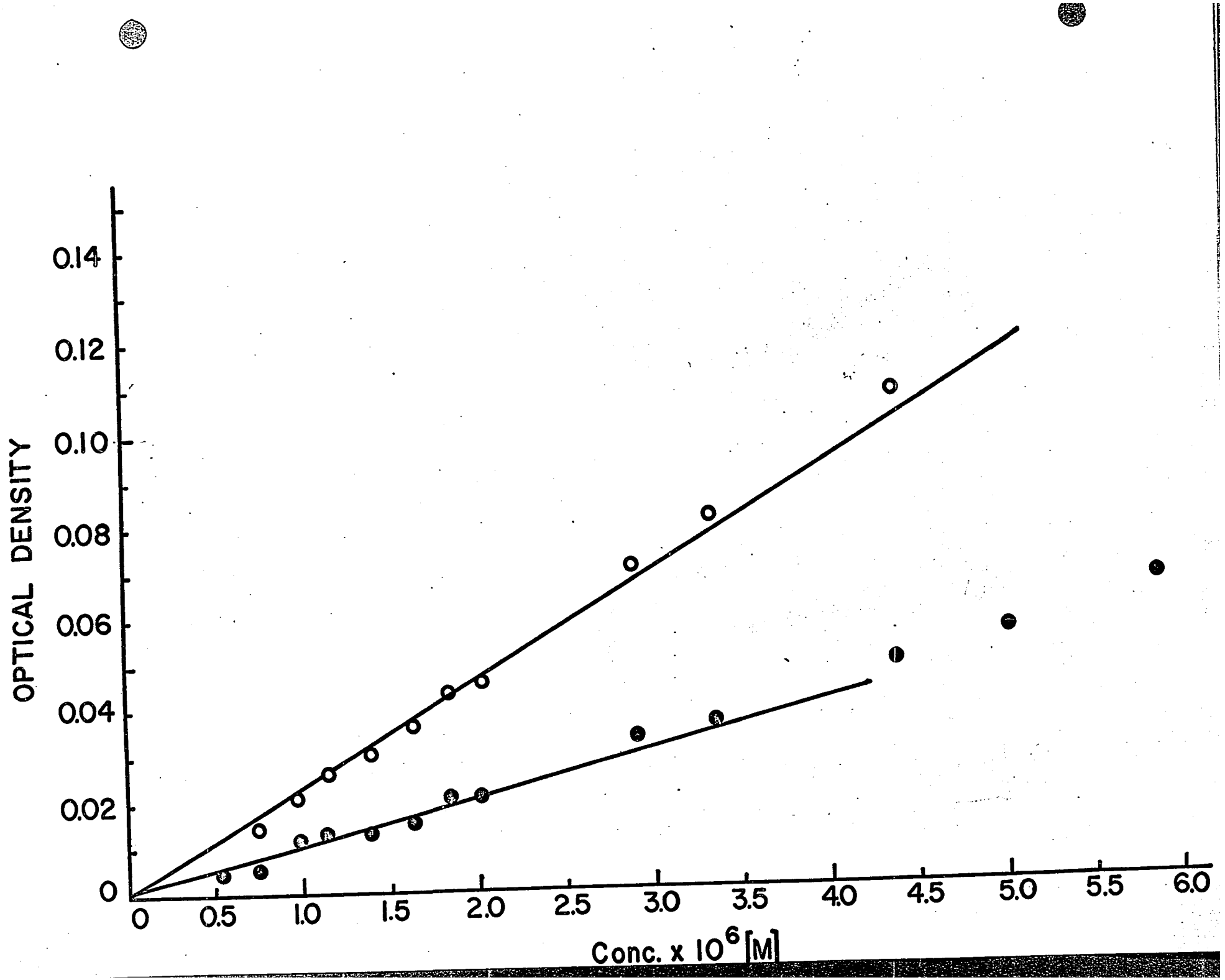

0.5
0.4
0.3
0.2
0.1
0
OPTICAL DENSITY
1
2
2
400
500
600
700
WAVELENGTH (mμ)

Figure 11

Determination of ϵ_b at pH 5, μ= 0.02 at 480 (-●-●-●-) and 600 mμ (-0-0-0-) in the IN-2S-4DNP——BSA system


OPTICAL DENSITY
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
Conc. x 10⁶ [M]

The values determined for the extinction coefficients of the bound and free forms of the dye, ϵ_b, and ϵ_f respectively, are shown in Table III.

Table III

	480 mμ	600 mμ
$\epsilon_f \times 10^{-4}$ l/mole cm	3.59	0
$\epsilon_b \times 10^{4}$ l/mole cm	0.98	2.33

It is obvious from Fig. 11 that the greater optical density values at 600 mμ allowed ϵ_b to be determined with greater precision at this wavelength than at 480 mμ. The value of ϵ_b at 600 mμ was therefore used to calculate the binding data for this system. As will also be noted from Table III the value of ϵ_f at 600 mμ is zero. This therefore allows calculation of the concentration of bound dye, b, present with a simplified form of formula (1), Chapter I, i.e.

$$b = \frac{\text{Optical Density (600 m}\mu\text{)}}{\epsilon_B \text{ 600 m}\mu}$$

The binding data calculated for this interaction* are listed in Table IV and are shown in the form of a $1/b$ vs $1/c$ plot in Figs12. It is also worth mentioning that the binding data calculated from readings at 480 mμ using equation (1) were found to be within a few percent of those calculated at 600 mμ. The values for K_o and the number of binding sites per molecule of BSA found by this graphical method were $2.3 \pm 0.3 \times 10^5$ M^{-1} and 4 ± 0.4 respectively. The errors in these values, estimated on the basis of the best upper and lower lines through the plotted data,

* Protein solutions used were 3.63×10^{-6} M

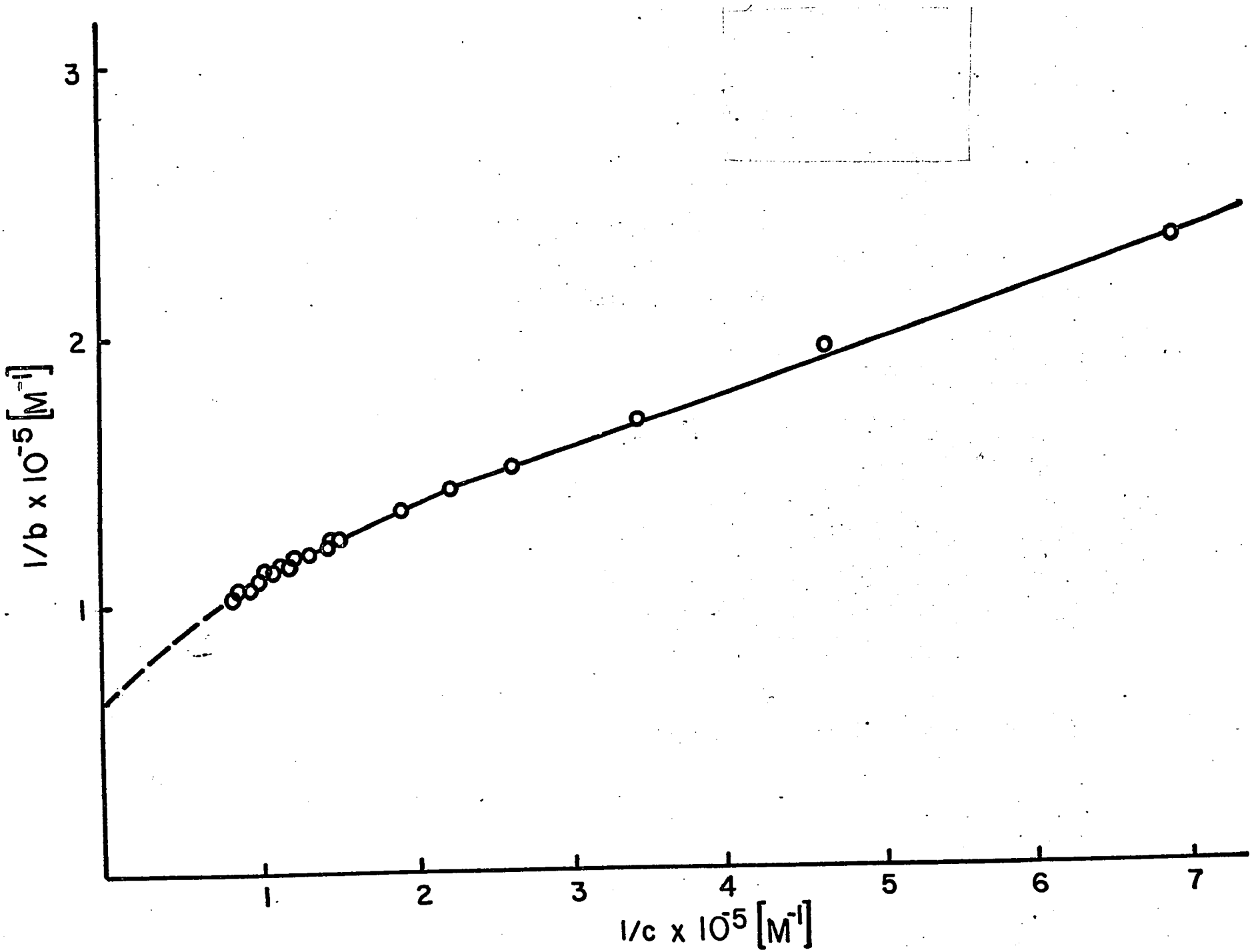
Table IV

Binding Data for the Reaction of IN-2S-4DNP with BSA

O.D. 600 mμ	b $\times 10^{-6}$ [M]	1/b $\times 10^{6}$ [M^{-1}]	Total IN-2S-4DNP added $\times 10^{6}$ [M]	c $\times 10^{-6}$ [M]	1/c $\times 10^{6}$ [M^{-1}]
0.081	3.47	0.288	4.32	0.85	1.176
0.100	4.29	0.233	5.74	1.45	0.690
0.120	5.15	0.194	7.30	2.15	0.465
0.140	6.01	0.166	8.91	2.90	0.345
0.155	6.65	0.150	10.7	4.05	0.247
0.164	7.04	0.142	11.5	4.46	0.224
0.174	7.47	0.134	12.7	5.23	0.191
0.188	8.07	0.124	14.7	6.63	0.151
0.190	8.15	0.123	15.0	6.85	0.146
0.192	8.24	0.121	15.2	6.96	0.144
0.198	8.50	0.118	16.0	7.50	0.133
0.199	8.54	0.117	16.8	8.26	0.121
0.204	8.76	0.114	17.0	8.24	0.121
0.205	8.80	0.114	17.5	8.70	0.115
0.208	8.93	0.112	18.1	9.17	0.109
0.210	9.01	0.111	18.6	9.59	0.104
0.212	9.10	0.110	18.6	9.50	0.105
0.214	9.18	0.109	19.0	9.82	0.102
0.217	9.31	0.107	19.7	10.39	0.096
0.222	9.53	0.105	20.7	11.22	0.089
0.226	9.70	0.103	21.6	11.90	0.084

Figure 12

Binding data for the 1N-2S-4DNP———BSA system at pH 5, μ= 0.02


3
2
1
1/b x 10^-5 [M^-1]
1
2
3
4
5
6
7
1/c x 10^5 [M^-1]

reflect the uncertainties inherent in the extrapolation of such a binding curve. It should be noted that values obtained at the lowest values of $1/_b$ and $1/_c$ are of solutions containing a large excess of dye. In this range of dye concentrations, any increase in the amount of dye added leads only to a small change in the amount of dye bound; therefore, these measurements are done under conditions of lowest sensitivity.

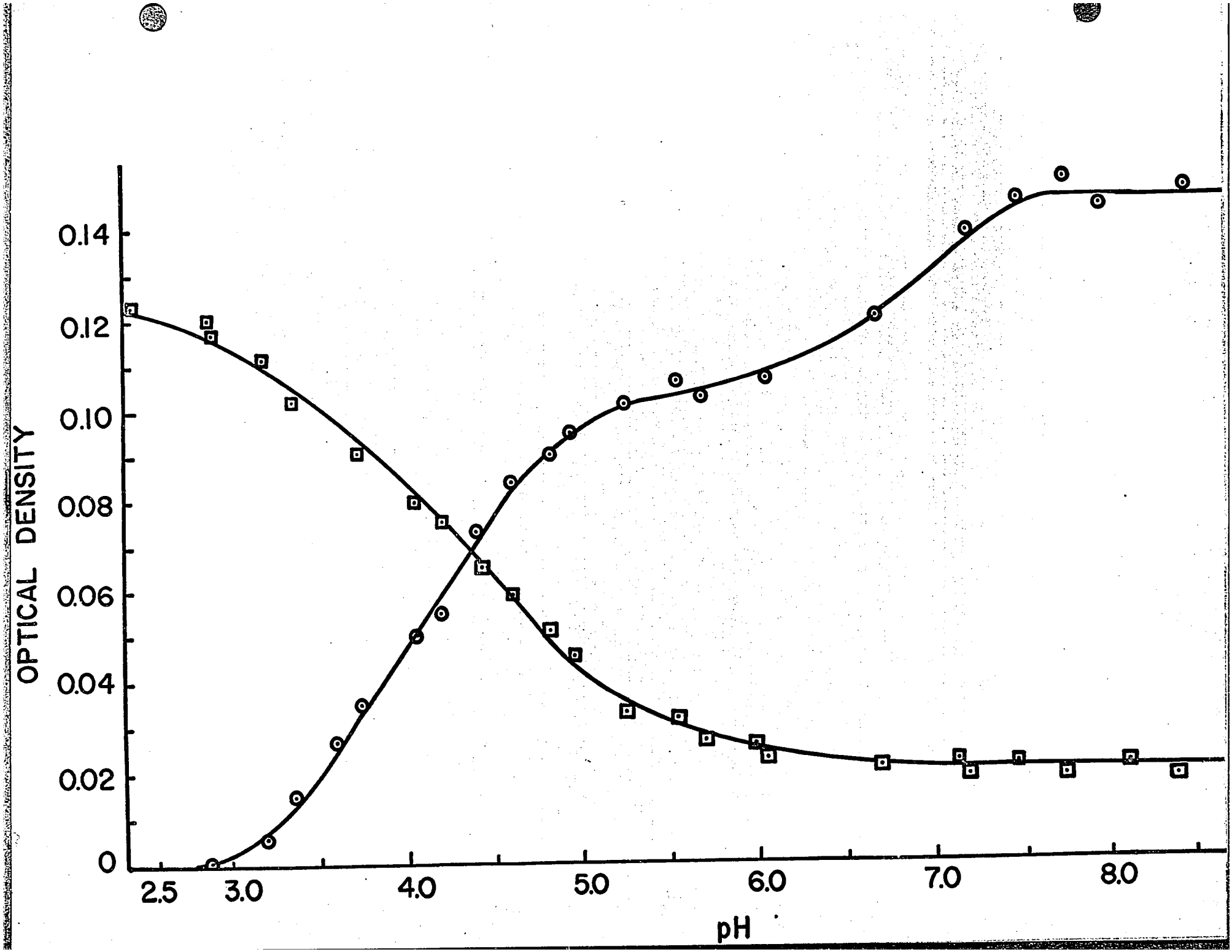
As can also be seen from Fig. 12, the slight curvature exhibited by this plot indicates that the binding sites of BSA for IN-2S-4DNP are not completely homogeneous. In Chapter III of this thesis the possibility of heterogeneity in antibody binding affinities leading to a variation in values of ϵ_b among different binding sites, will be discussed. In view of this it is possible that the binding data determined here might also deviate from true values obtainable by equilibrium dialysis experiments. However, such a comparison could not be made due to the tendency of IN-2S-4DNP to bind strongly to dialysis membranes under the experimental conditions of this study.

Effect of pH on the IN-2S-4DNP———BSA Reaction

As the binding data calculated at 480 and 600 mμ indicated that IN-2S-4DNP was bound to BSA in two forms, which were in equilibrium with one another, experiments were performed to determine whether the ratio of these forms depended on pH as well as whether variations in this parameter would affect the extent of dye binding by the protein. The effect of varying the pH on the optical densities at 480 and 625 mμ of the bound dye is shown in Fig. 13. The concentration of BSA in the solution titrated was kept sufficiently high (5×10^{-4} M) to ensure that all the dye present (4.2×10^{-6} M) was bound to the protein.

Figure 13

Spectrophotometric titration of IN-2S-4DNP bound to BSA. -0-0-0 - 625 mμ; -⊡-⊡- 480 mμ


OPTICAL DENSITY
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
2.5
3.0
4.0
5.0
6.0
7.0
8.0
pH

To determine the apparent pK of the ionization process occurring in the pH range of from 2 to 6, extinction coefficients for the two bound forms of the dye were calculated at 480 and 625 mμ from this plot. These values were then used to determine the degree of dissociation of the bound dye, α, from the expression

$$\alpha = \frac{\epsilon_{pH} - \epsilon_2}{\epsilon_6 - \epsilon_2}$$

where ϵ_{pH} is the extinction coefficient at the pH at which α is to be evaluated and ϵ_6, ϵ_2 are the extinction coefficients at pH 6 and 2, all at the same wavelength. The values obtained for α calculated at both 480 and 625 mμ were then plotted in the form of $\log \frac{\alpha}{1-\alpha}$ vs pH [Fig. 14]. The fact that the data obtained at both wavelengths fall on the same straight line indicates that a single ionization process, with an apparent pK of 4.3*, occurs in this pH range.

Another process that leads to an increase of the extinction coefficient of the bound dye at 625 mμ also appears to occur in the pH range of from 6 to 7.5. Although this phenomenon was not considered in any detail in this study it is obvious that it cannot be related to a further ionization of the bound dye. BSA is not known to undergo any significant conformational change in this pH range (123). However, it is still possible that titration of the protein produces a small structural alteration that leads to a stabilization of the bound dye in a form characterized by a greater extinction coefficient.

The spectra of the BSA-dye solutions (used in the pH titration experiments) at various pH values are shown in Fig. 15. As

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* The value at which $\log \frac{\alpha}{1-\alpha} = 0$ corresponds to a value of α equal to 1/2. By definition the pH at which a compound is half dissociated is taken as its pK.

Figure 14

Determination of the pK of IN-2S-4DNP bound to BSA; ●-480 mμ, ⊙-625 mμ.

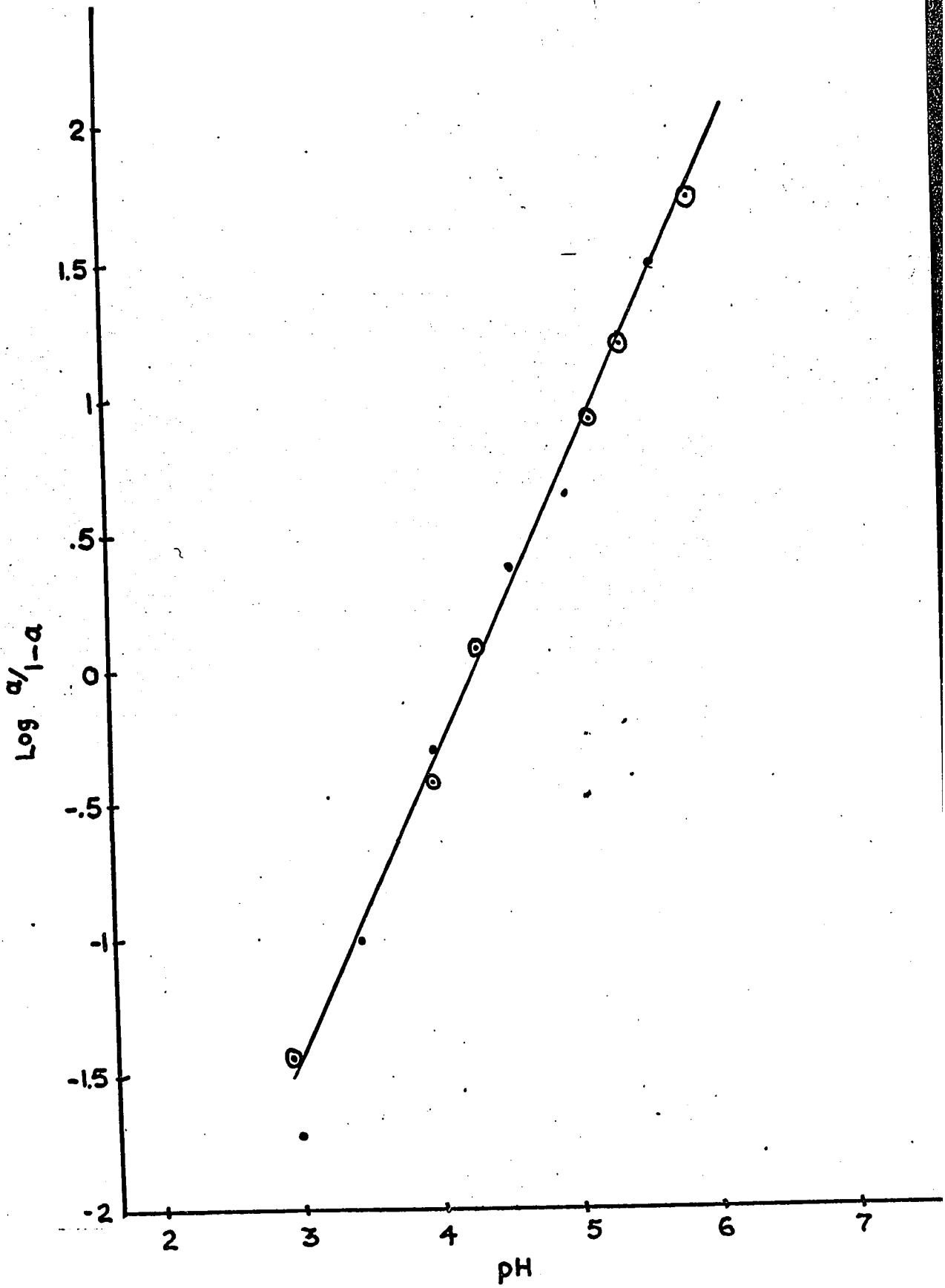

2
1.5
1
.5
0
-.5
-1
-1.5
-2
Log a/1-a
2
3
4
5
6
7
pH

Figure 15

Spectrum of 1N-2S-4DNP bound to BSA at different pH'S, Curve #1 - pH 2.9, Curve #2 - pH 4.1, Curve #3 - pH 5, Curve #4 - pH 6, Curve #5 - pH 7.8

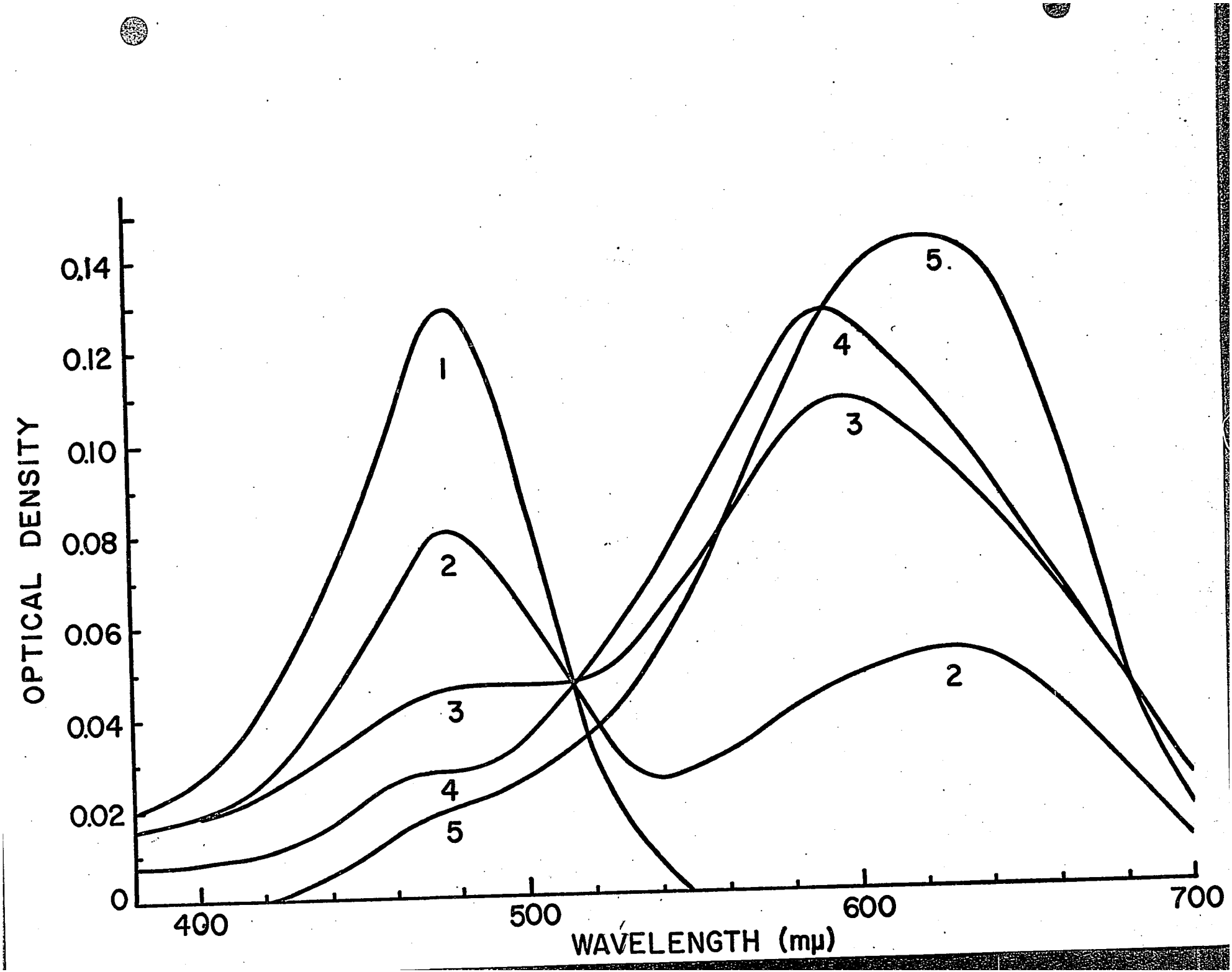

OPTICAL DENSITY
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
400
500
600
700
WAVELENGTH (mμ)
1
2
3
4
5
5
4
3
2

Figure 16

Chromatogram of a BSA———IN-2S-4DNP mixture and of IN-2S-4DNP at pH 2, μ = 0.02. Curve #1 and Curve #2 - absorbance of mixture at 280 and 480 mμ respectively. Curve #3 - absorbance of dye solution at 480 mμ run under identical conditions

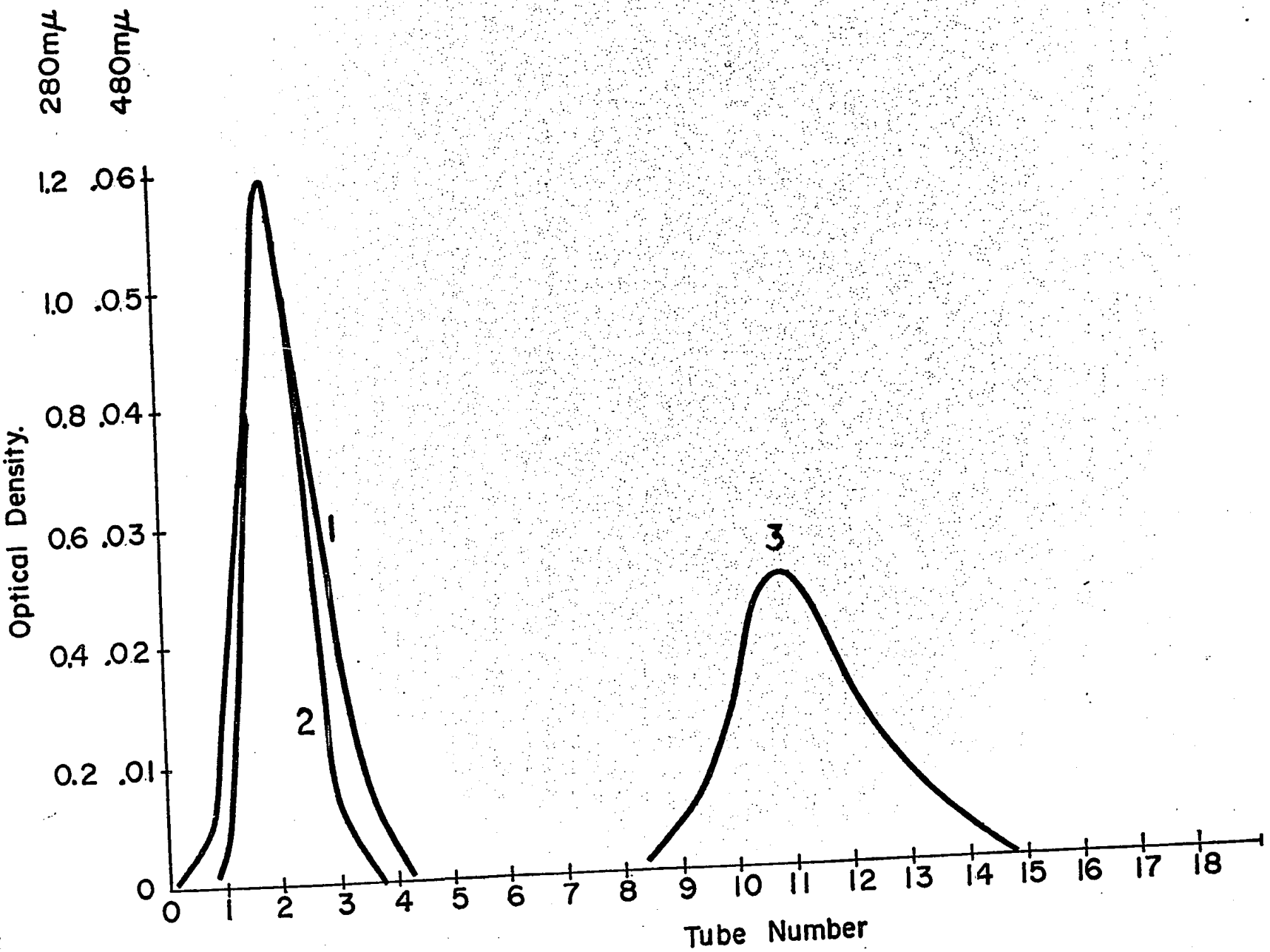
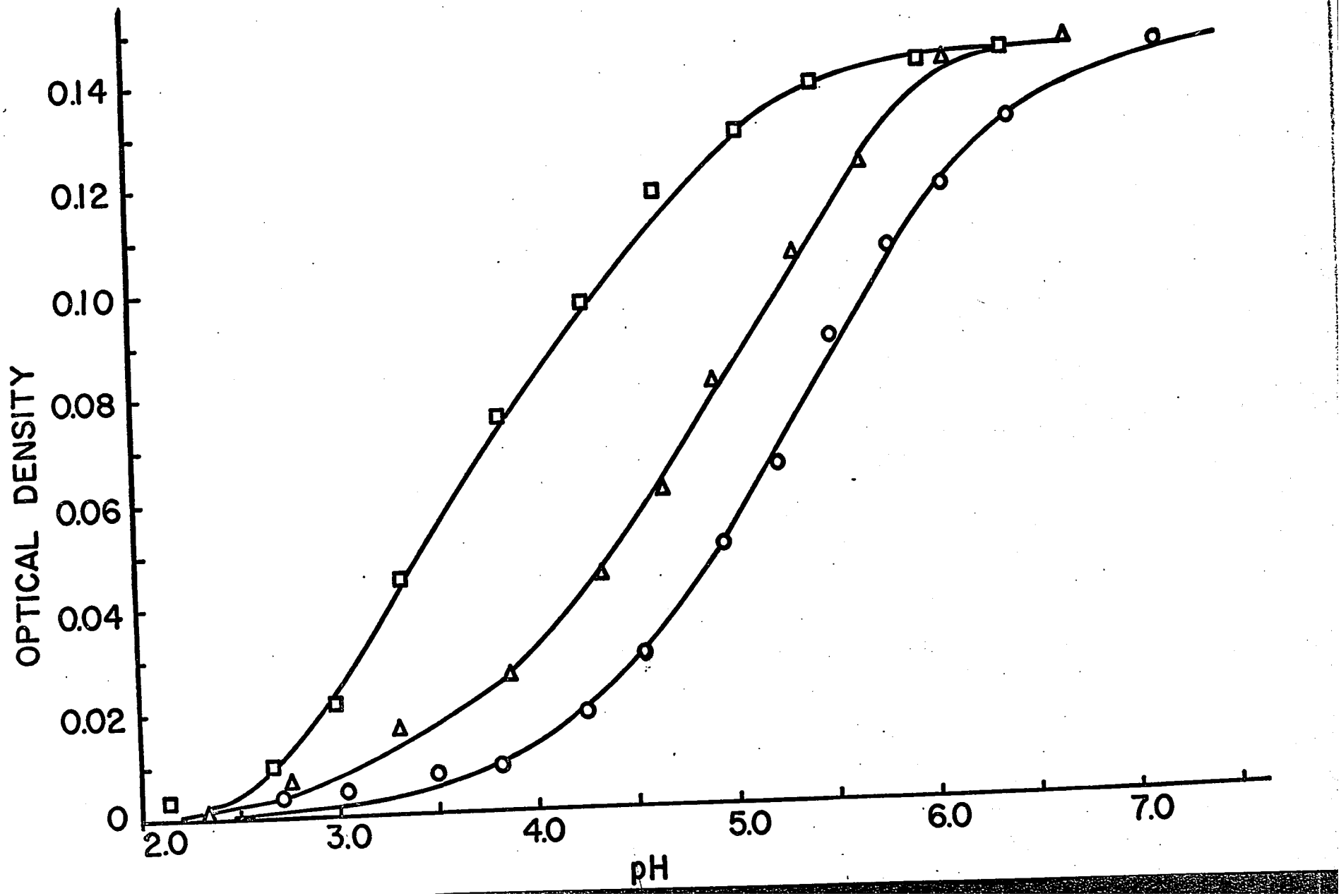

280mμ
480mμ
1.2
.06
1.0
.5
0.8
.4
0.6
.03
0.4
.02
0.2
.01
0
Optical Density.
1
2
3
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Tube Number

Figure 17

Spectrophotometric titration of BSA———IN-2S-4DNP mixtures in the presence of different ions. ▫-▫ deionized water, ▵-▵ $NaNO_3$ (μ= 0.02), 0-0-0 NaSCN (μ = 0.02)


OPTICAL DENSITY
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
2.0
3.0
4.0
5.0
6.0
7.0
pH

can be seen, addition of BSA to the dye at pH 2 does not cause a spectral shift, although a decrease in extinction coefficient does occur. To ensure that this was not a non-specific effect due, for example, to an increase in dielectric constant of the medium at high protein concentration, and to verify the conclusion drawn from the results of the pH titration, that the disappearance of the spectral shift was due to protonation of the bound dye and not to its dissociation from the protein, a solution that was 3.63×10^{-6} M in BSA and 4.2×10^{-6} M in IN-2S-4DNP was examined by gel filtration at pH 2, μ = 0.02. The dye was found to migrate with the protein [Fig. 16] indicating that it was strongly bound to BSA at this low pH.

The titration curve of bound IN-2S-4DNP in the pH range of from 2 to 6 [Fig. 13] occurs in a similar range to that of the carboxyl groups of the native protein (123). As the presence of anions has a well known effect on the latter, (123) experiments were done to compare the effect of thiocyanate and nitrate ions on the titration curve of IN-2S-4DNP bound to BSA. In Fig. 17 are shown the titration curves of a BSA-dye solution (3.63×10^{-6} M in BSA and 4.5×10^{-6} M in IN-2S-4DNP) in (i) deionized water, in (ii) a 0.02M solution of sodium thiocyanate and in (iii) a 0.02M solution of sodium nitrate, all at 625 mμ.

The presence of these anions is seen to produce a marked shift of the titration curves to higher pH values, the shift being greater for the more firmly bound thiocyanate anion.* Thus the effect of these anions on the titration curve of the protein's carboxylate groups and

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* It will be noted that a slight difference exists between the titration curves performed in the absence of ions in Figs. 13 and 17. This was most likely due to an insufficient excess of protein and therefore a small amount of free dye being present in the latter.

on that of bound IN-2S-4DNP molecules, would appear to be very similar. In both cases binding of anions leads to an increase in the protein's negative charge and therefore to a greater affinity of the protein for protons. In the case of a protein-dye complex increased proton binding will also lead to "titration" of any ionized bound dye molecules and therefore to a smaller spectral shift.

The Effect of Inhibitors on the BSA ——— IN-2S-4DNP Reaction

From considerations of the structural formula of IN-2S-4DNP the binding of this dye to BSA would be thought to involve one or both of the two main portions of the dye molecule i.e. the napthol sulfonic residue and the 2, 4-dinitrophenyl group. With this in mind it was decided to investigate the effect of various related molecules, such as napthol sulfonic acids and nitro-phenols, on the reaction of this dye with BSA, to ascertain whether an excess of these reagents could displace the bound dye from the protein. At the same time the effects of related azo dyes, i.e. IN-2S-4pNP and IN-3, 6S-2DNP, as well as of reagents such as urea and dioxane, whose effects on reactions between proteins and small molecules have been demonstrated in other systems, (176, 177) were investigated. As the appearance of a spectral shift in the binding of IN-2S-4DNP to BSA provided a simple means of determining that interaction had occurred in this system, the relative inhibitory effects of the various reagents used were judged on the basis of their effect on the color change.

All comparisons were made at pH 5, μ = 0.02 in solutions that were 3.63×10^{-6} M and 4.5×10^{-6} M in BSA and IN-2S-4DNP, respectively.

Under these conditions (in the absence of any inhibitor) about 80% of the dye present was bound to the protein.

A) Napthol Sulfonic Acids and Napthol

The effect of adding (i) 1-napthol 2-sulfonic acid, (ii) 1-napthol 4-sulfonic acid, (iii) 1-napthol 3, 6-disulfonic acid and (iv) 1-napthol, in different concentrations, to a solution of BSA and 1N-2S-4DNP is shown in Figs. 18 - 21 respectively.

A comparison of these figures indicates that both the 1, 2- and 1, 4-napthol sulfonic acids were about ten times as effective as either the 1-Napthol 3, 6-disulfonic acid or 1-Napthol in inhibiting the spectral shift of 1N-2S-4DNP.

B) Nitrophenols

The inhibitory effects of 2- and 4-nitrophenol and of 2, 4-dinitrophenol on the 1N-2S-4DNP spectral shift at pH 5 are depicted in Figs. 22 and 23 respectively. Comparison of these figures with figures 18 and 19 shows that the dinitrophenol molecule was as effective as either of the napthol mono-sulfonic acids in reducing the spectral shift. On the other hand the mono-nitrophenols, whose relatively small solubilities prevented the use of higher concentrations of these substances, had almost no effect on the reaction. It should be noted that the pK of 2, 4-dinitrophenol is 3.96 while that of both 2- and 4- nitrophenol is 7.16. At pH 5, therefore, almost all the dinitrophenol present will be ionized, while the mononotrophenols will be in the form of neutral molecules.

Figure 18

Effect of 1-Napthol 2-sulfonic acid (IN-2S) on a BSA———IN-2S-4DNP solution. Curve #1 - Buffer only pH 5, μ= 0.02, Curve #2 - Buffer plus 3.6×10^{-4} M IN-2S, Curve #3 - 3.6×10^{-3} M IN-2S, Curve #4 - 3.6×10^{-2} M IN-2S.

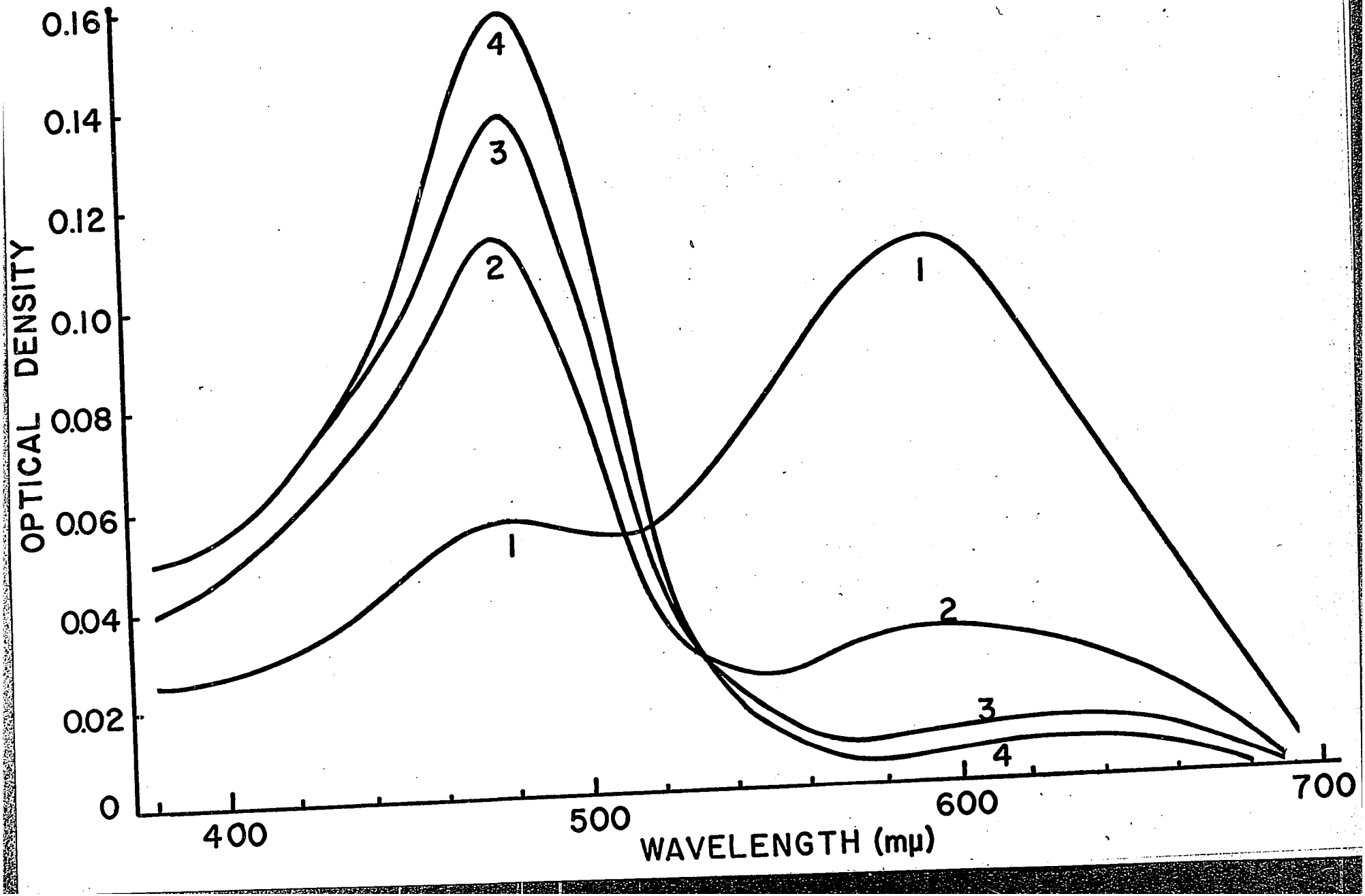

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
4
3
2
1
1
2
3
4
400
500
600
700
WAVELENGTH (mμ)

Figure 19

Effect of 1-Napthol 4-sulfonic acid (1N-4S) on a BSA———1N-2S-4DNP solution. Curve #1 - Buffer only pH 5, μ = 0.02, Curve #2 - Buffer plus 2.4×10^{-4} M 1N-4S, Curve #3 - Buffer plus 12×10^{-3} M 1N-4S.

Figure 19

Effect of 1-Napthol 4-sulfonic acid (IN-4S) on a BSA———IN-2S-4DNP solution. Curve #1 - Buffer only pH 5, μ = 0.02, Curve #2 - Buffer plus 2.4×10^{-4} M IN-4S, Curve #3 - Buffer plus 12×10^{-3} M IN-4S.

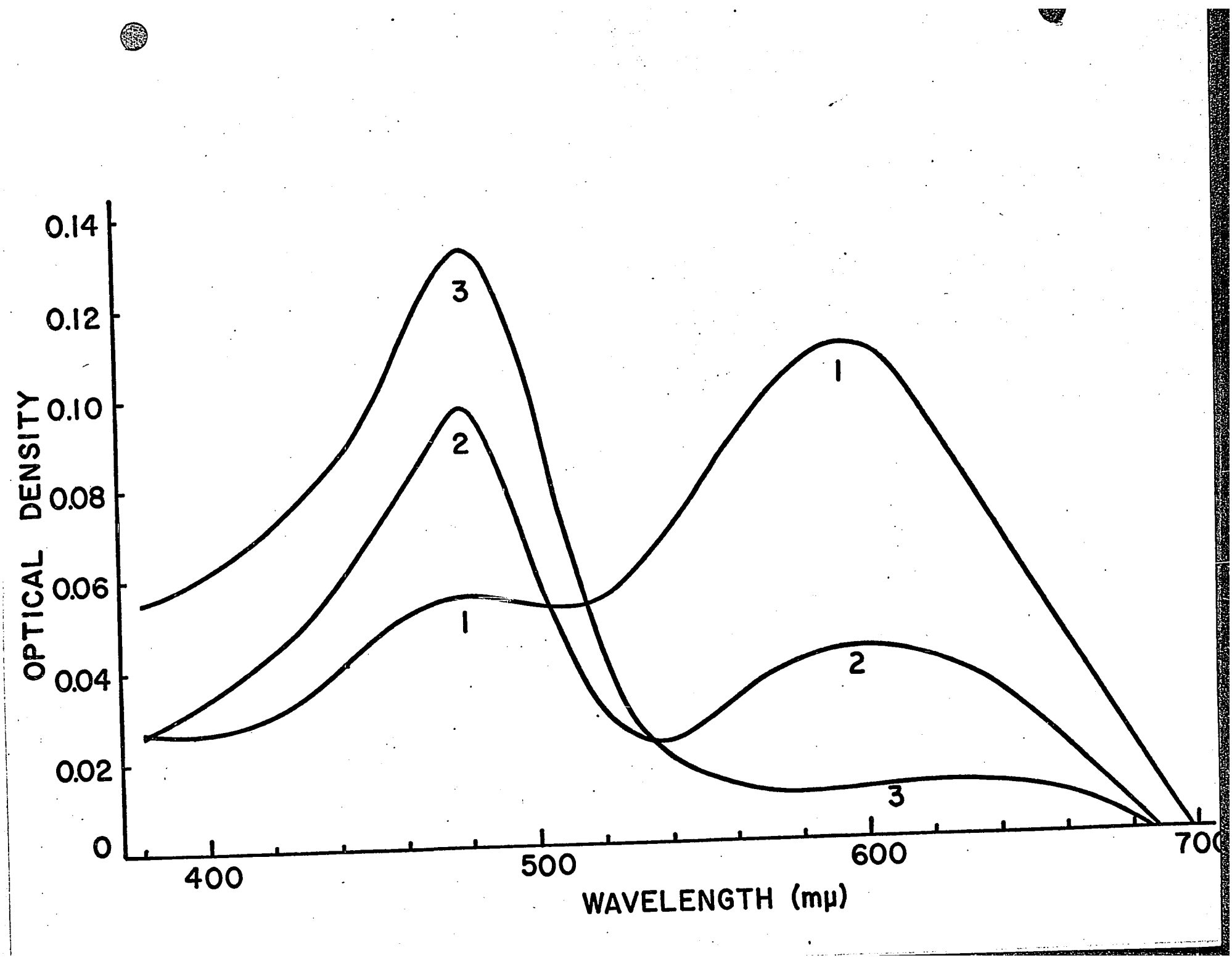

0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
400
500
600
700
WAVELENGTH (mμ)
1
2
3
1
2
3

Figure 20

Effect of 1-Napthol 3, 6-disulfonic acid (1N-3, 6S) on a 1N-2S-4DNP———BSA solution. Curve #1 - Buffer only (pH 5, μ = 0.02), Curve #2 - Buffer plus 3.6×10^{-3} M 1N-3, 6S, Curve #3 - Buffer plus 18×10^{-3} M 1N-3, 6S, Curve #4 - Buffer plus 36.5×10^{-3} M 1N-3, 6S.

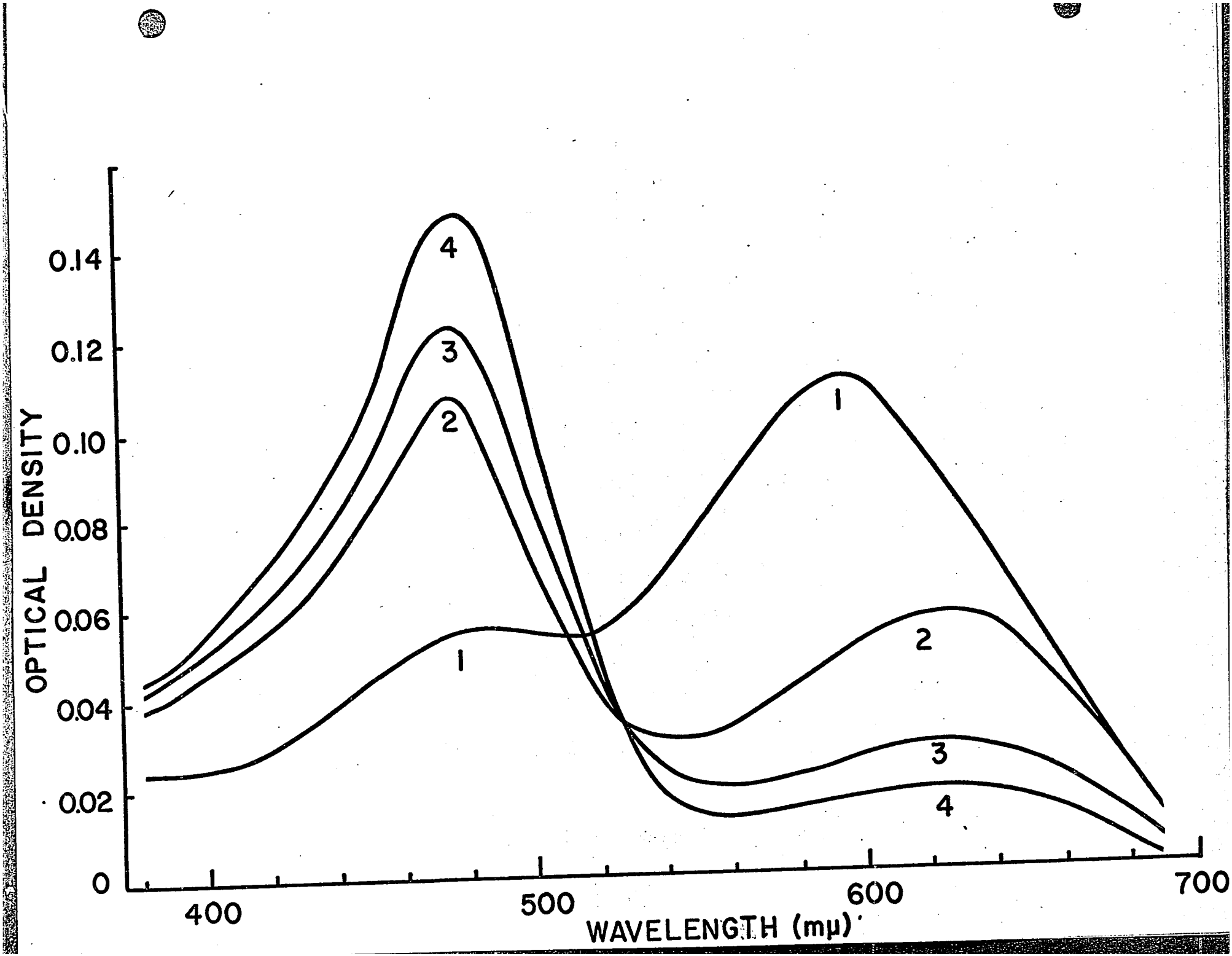

OPTICAL DENSITY
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
400
500
600
700
WAVELENGTH (mμ)
1
2
3
4
4
3
2
1
2
3
4

Figure 21

Effect of 1-Napthol on a 1N-2S-4DNP———BSA solution. Curve #1 - Buffer only pH 5, μ= 0.02, Curve #2 - Buffer plus 3×10^{-3} M 1-Napthol.

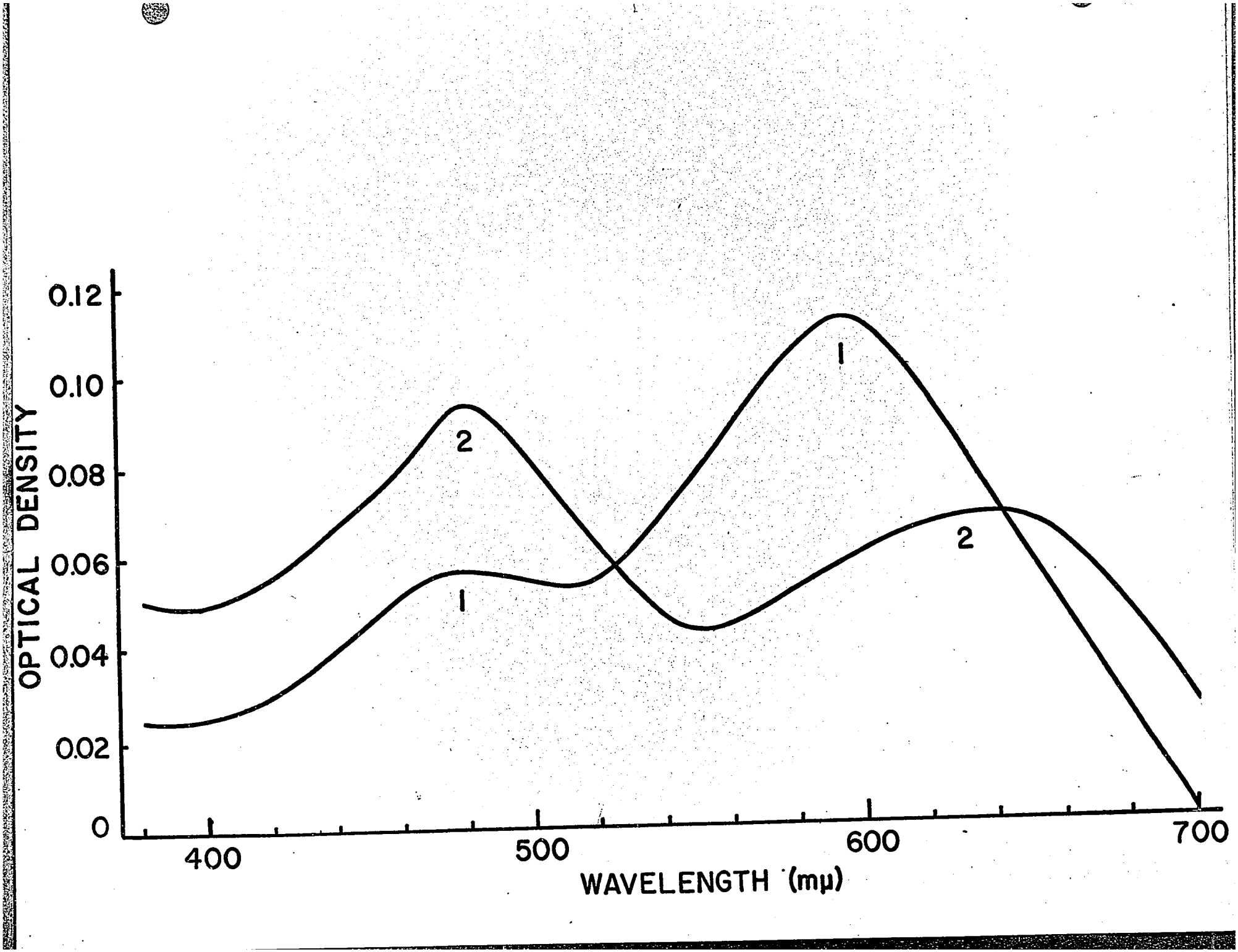

0.12
0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
400
500
600
700
WAVELENGTH (mμ)
1
2
1
2

Figure 22

Effect of ortho and para Nitrophenol on a IN-2S-4DNP ——BSA solution. Curve #1 - Buffer only pH 5, μ= 0.02, Curve #2 - Buffer containing 3×10^{-3} M p-Nitrophenol, Curve #3 - Buffer containing 4×10^{-3} M o-Nitro phenol.

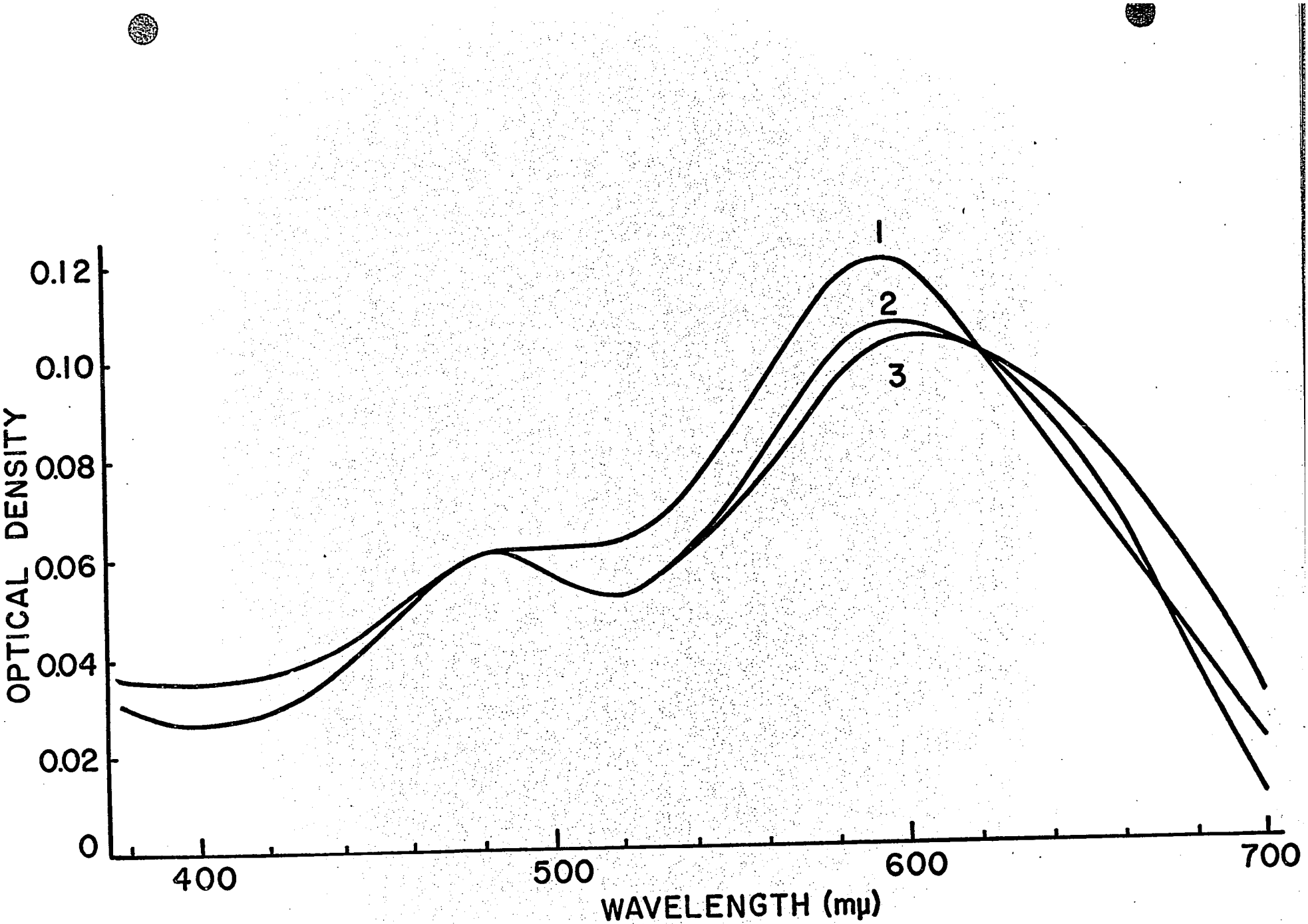
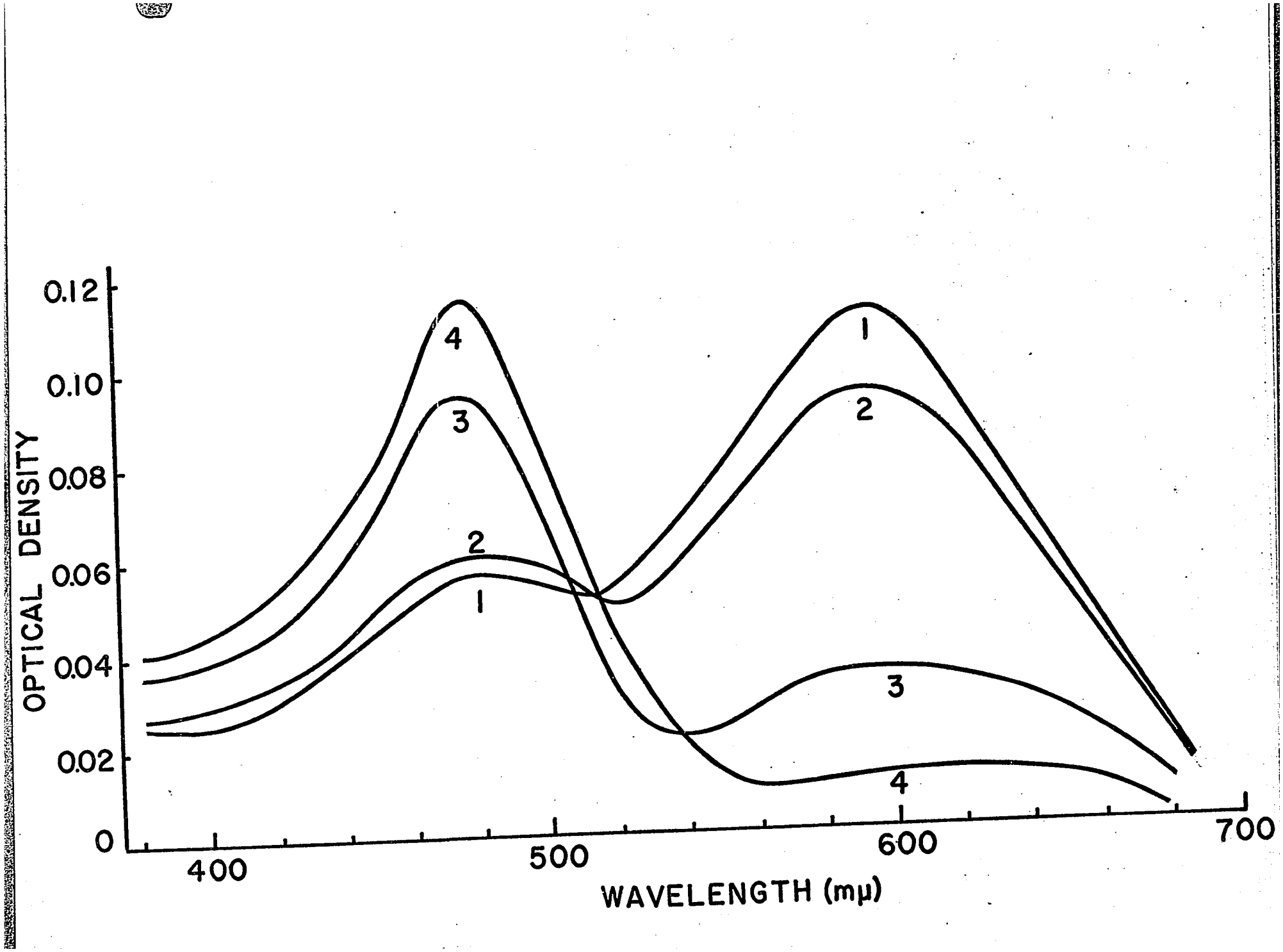

0.12
0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
400
500
600
700
WAVELENGTH (mμ)
1
2
3

Figure 23

Effect of 2, 4-Dinitrophenol (2, 4-DNP) on a 1N-2S-4DNP ———BSA solution. Curve #1 - Buffer only pH 5, μ= 0.02, Curve #2 - Buffer plus 0.5×10^{-4} M 2, 4-DNP, Curve #3 - Buffer plus 5.3×10^{-4} M 2, 4-DNP, Curve #4 - Buffer plus 56×10^{-4} M 2,4-DNP.


0.12
0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
400
500
600
700
WAVELENGTH (mμ)
1
2
3
4
1
2
3
4

C) Azo-Dyes

The result of adding IN-2S-4pNP and IN-3, 6S-2DNP at final concentrations within a few percent of IN-2S-4DNP* present in the BSA solution is shown in Figs. 24 and 25. The effects of the two dyes differ considerably; IN-3, 6S-2DNP appears to act as a competitive inhibitor as it causes a decrease in absorption at 600 mμ along with a proportional increase in the absorption of the dye at 480 mμ. This effect was not very great; in fact a reduction of approximately 10% in optical density at 600 mμ was observed.

Calculations performed on the basis of the assumption that IN-3, 6S-2DNP had the same affinity for the protein as IN-2S-4DNP predicted that the addition of the former dye would lead to a decrease in absorption at 600 mμ of about the magnitude found. However it should be noted that the possibility that this is a non-specific effect, due to the increase in protein charge through the binding of the anionic IN-3, 6S-2DNP cannot be eliminated on the basis of the experiments reported.

In the case of IN-2S-4pNP [Fig. 24], a reduction in absorption at both 480 and 600 mμ occurred. This effect cannot be attributed to a simple competition or charge effect and may be due to the binding of this dye causing a conformational change in the protein, that in turn alters the spectrum of the bound form of IN-2S-4DNP.

D) Glycine, Dioxane, Urea

Of these three substances only dioxane had any measurable effect

*The concentration of IN-2S-4pNP was estimated on the basis of the assumption that its extinction coefficient was identical to that of IN-2S-4DNP.

Figure 24

Effect of IN-2S-4pNP (4×10^{-6} M) on a IN-2S-4DNP——BSA solution. Curve #1 - Buffer only pH 5, μ= 0.02, Curve #2 - Buffer plus IN-2S-4pNP.

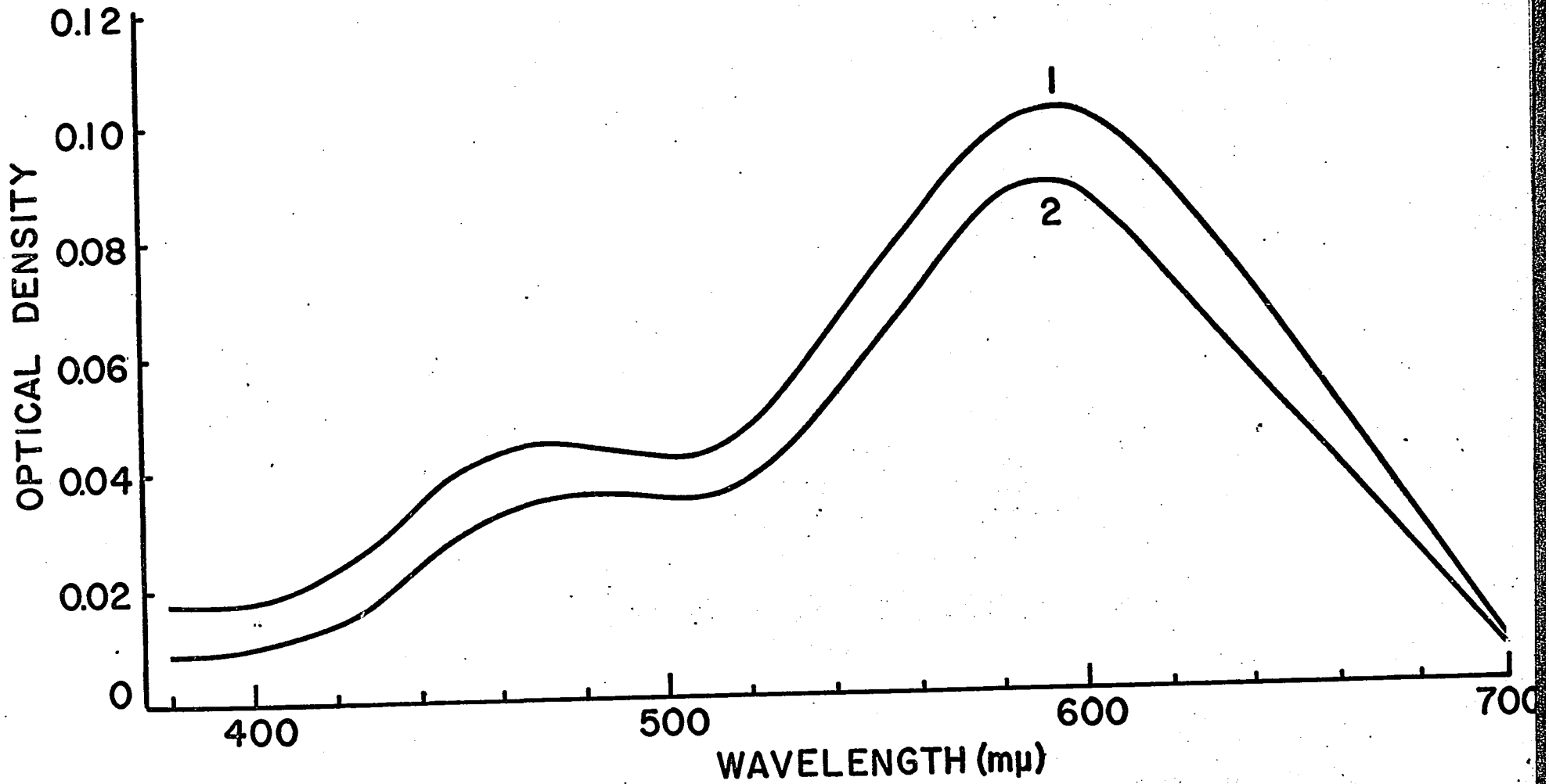
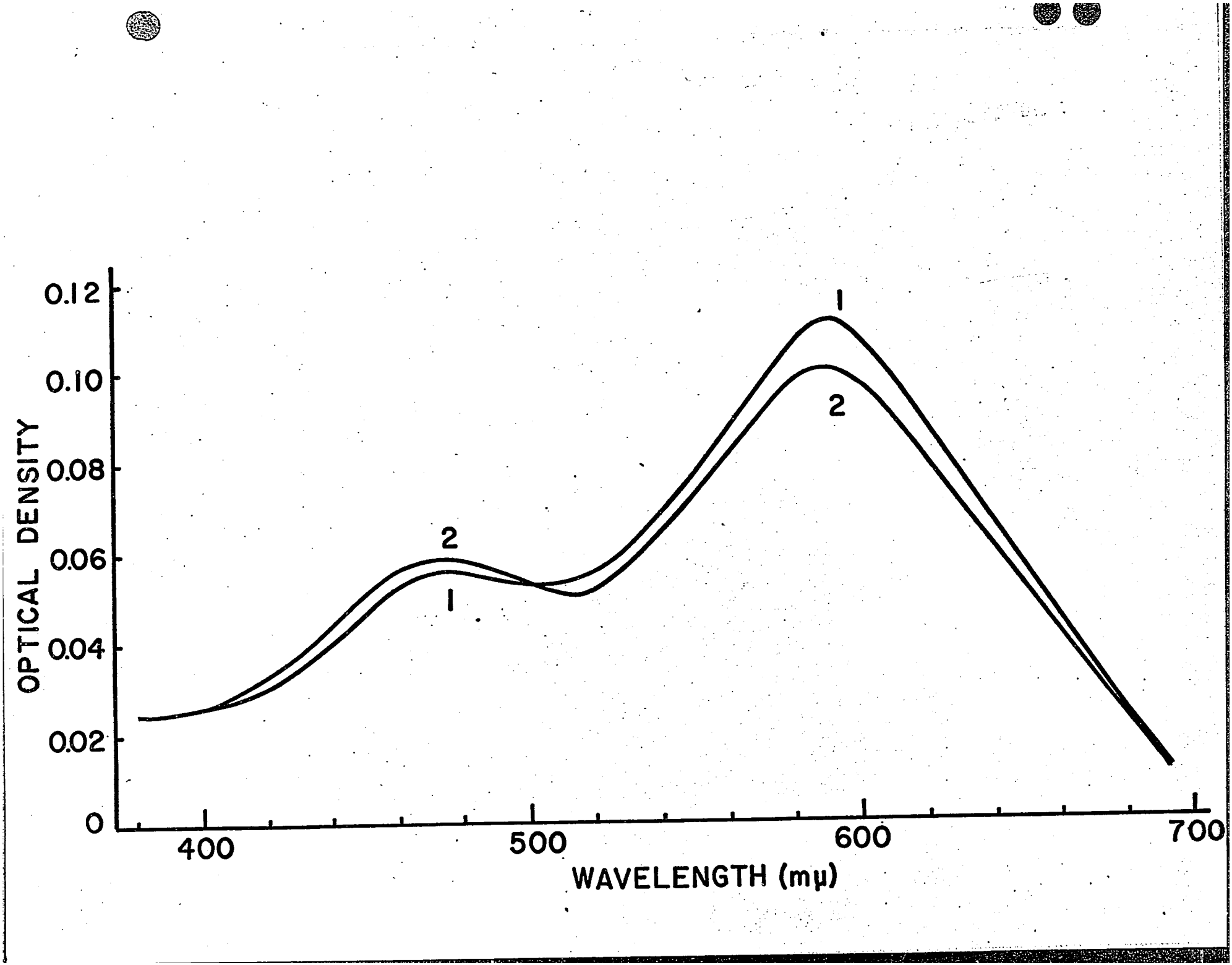

0.12
0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
1
2
400
500
600
700
WAVELENGTH (mμ)

Figure 25

Effect of IN-3, 6S-2DNP on the spectrum of a IN-2S-4DNP ——— BSA solution (at pH 5, μ= 0.02). Curve #1 - Buffer only, Curve #2 - Buffer plus 4×10^{-6} M IN-3, 6S-2DNP.


0.12
0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
400
500
600
700
WAVELENGTH (mμ)
1
2
2
1

on the spectrum of the free dye; this effect is shown in Fig. 26, for solutions of IN-2S-4DNP in 20% dioxane at pH 5 and 9.5 respectively.

The addition of glycine (to a final concentration of 1.4 M) to a BSA ——— IN-2S-4DNP mixture had little effect on the spectrum of the bound dye [Fig. 27]. As this reagent causes a large increase in dielectric constant of the medium, its small effect may be interpreted in terms of electrostatic interactions being relatively unimportant in the IN-2S-4DNP-BSA reaction.

The effect of dioxane at concentrations of 10 and 20%, on the spectrum of bound IN-2S-4DNP is shown in Fig. 28. The large increase in absorption at both 480 and 600 mμ caused by the reagent cannot be due to a displacement of the dye from its binding sites on the protein or to a change in the state of ionization of the bound dye. The most likely explanation then, is that the addition of dioxane causes some alteration in the BSA ———IN-2S-4DNP interaction, perhaps through a conformational change in the protein induced by dioxane, which in turn leads to the change in the spectrum of the bound dye.

The small effect of glycine has implicated the importance of hydrophobic bonding in this system, and, therefore, the effect of dioxane seems slightly anomalous. The addition of dioxane to a water solution lowers the dielectric constant and increases the solution's overall "organic" character. Thus the binding of an organic molecule to a protein would be expected to be less in a water-dioxane solution if the driving force for the reaction was the relative insolubility of the organic molecule in water as compared to the "organic milieu" of a protein binding site. The reaction of IN-2S-4DNP with BSA is evidently due to more specific and stronger forces than those assumed in such an analogy. Thus

Figure 26

Effect of dioxane (20%) on the spectrum of IN-2S-4DNP - Curve #1 - Buffer pH 5, μ = 0.02, Curve #2 - Buffer plus dioxane pH 5, Curve #3 - Buffer pH 9.5, μ = 0.02, Curve #4 - Buffer plus dioxane pH 9.5.

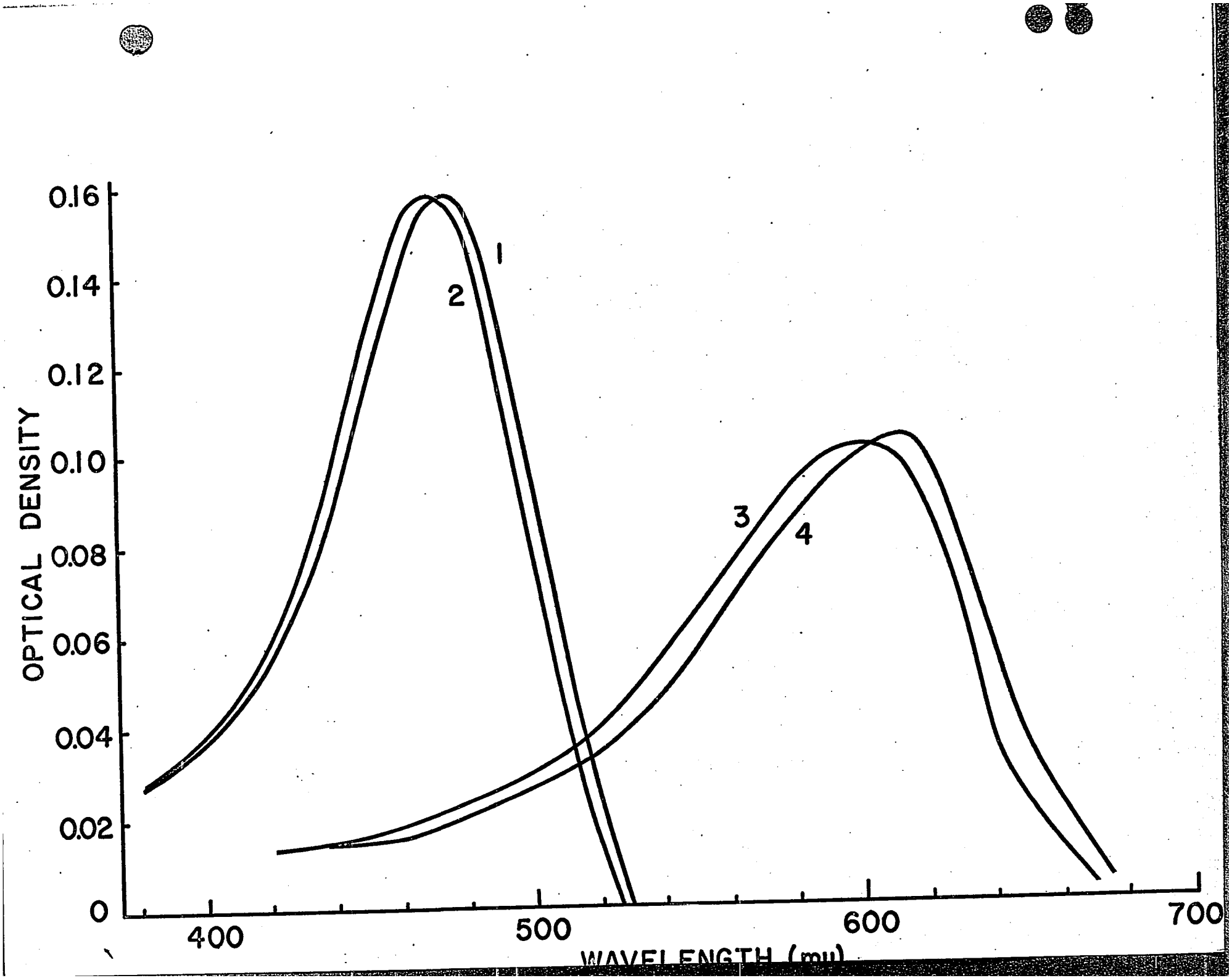

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
400
500
600
700
WAVELENGTH (mμ)
1
2
3
4

Figure 27

Effect of glycine (1.4 M) on the spectrum of a 1N-2S-4DNP——BSA solution (at pH 5, μ = 0.02). Curve #1 - Buffer only, Curve #2 - Buffer plus glycine.

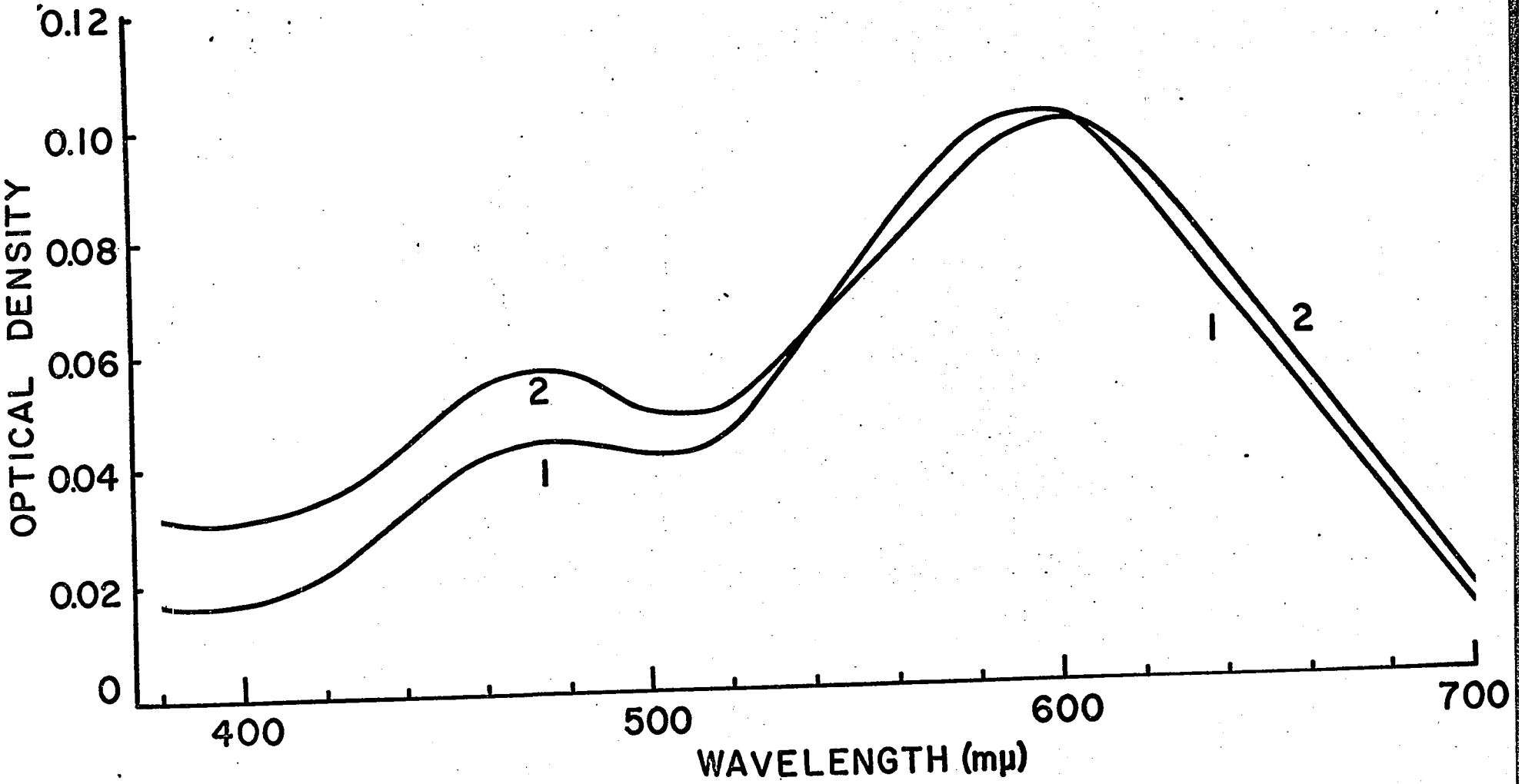
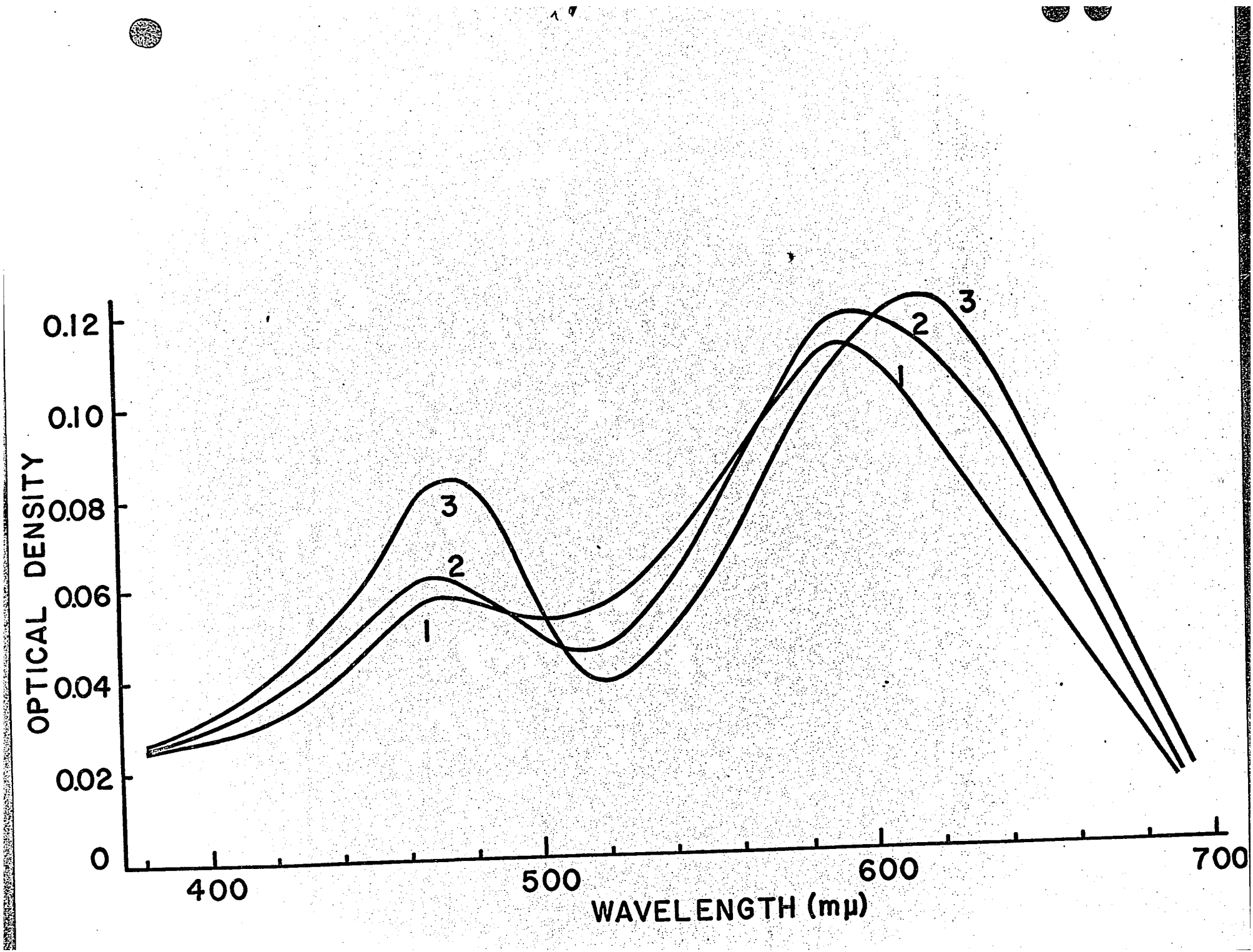

0.12
0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
400
500
600
700
WAVELENGTH (mμ)
1
2
2
1

Figure 28

Effect of dioxane on a 1N-2S-4DNP———BSA solution at pH 5, μ= 0.02. Curve #1 - Buffer only, Curve #2 - Buffer plus 10% dioxane, Curve #3 - Buffer plus 20% dioxane .


0.12
0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
400
500
600
700
WAVELENGTH (mμ)
1
2
3
1
2
3

the free energy change due to the binding of this dye to BSA cannot be accounted for solely on the basis of the molecule being in a region of lower dielectric constant.

In the presence of 7.5 M urea no spectral shift was observed in the IN-2S-4DNP——BSA system [Fig. 29]. Such an effect is in line with the ability of this molecule to disrupt protein structure (177) and consequently to destroy the binding sites of BSA for the dye. However, it is also possible, albeit unlikely in view of the differences in charge and structure, that the inhibition of the spectral shift caused by urea is due to a competitive phenomenon.

E) Acetate and Chloride Ion

The result of increasing the ionic strength of a BSA———IN-2S-4DNP solution to 0.04 by addition of acetate ion is shown in Fig. 30. The change occurring appears to be due to two effects. Thus the increase in absorption at 480 mμ would seem to result from a shift in the pK of the bound dye as occurred in the presence of other anions, i.e. thiocyanate. On the other hand the small shift of the absorption spectrum in the region of 600 mμ to higher wavelengths could be due to an alteration of the proteins structure under these conditions, which in turn alters the spectrum of the bound dye.

The effect of a greater increase in ionic strength on the interaction of IN-3, 6S-2DNP with BSA at pH 4.7 is much more dramatic, [Fig. 31]. As can be seen very little spectral shift occurred at an ionic strength of chloride ion equal to 0.1, in contrast to that observed at a lower ionic strength at this pH. This would account for the failure of

Figure 29

Effect of urea on a IN-2S-4DNP———BSA solution at pH 5, μ = 0.02. Curve #1 - Buffer only, Curve #2 - Buffer plus 5 M urea, Curve #3 - Buffer plus 7.5 M urea.

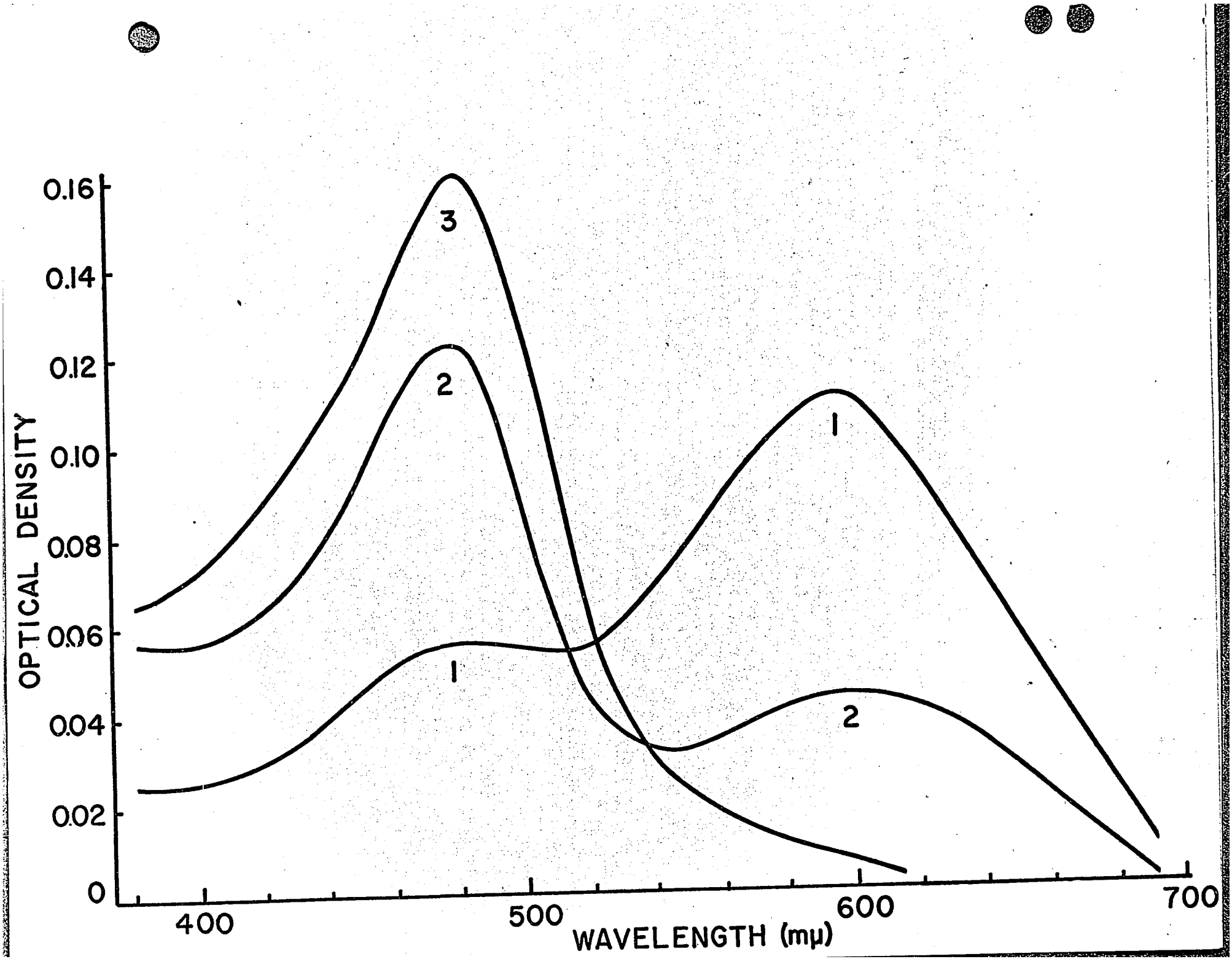

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
OPTICAL DENSITY
3
2
1
1
2
400
500
600
700
WAVELENGTH (mμ)

Figure 30

Effect of acetate ion on the spectrum of a 1N-2S-4DNP ——BSA solution. Curve #1 - μ = 0.02 acetate pH 5, Curve #2 - μ = 0.04 acetate pH 5.

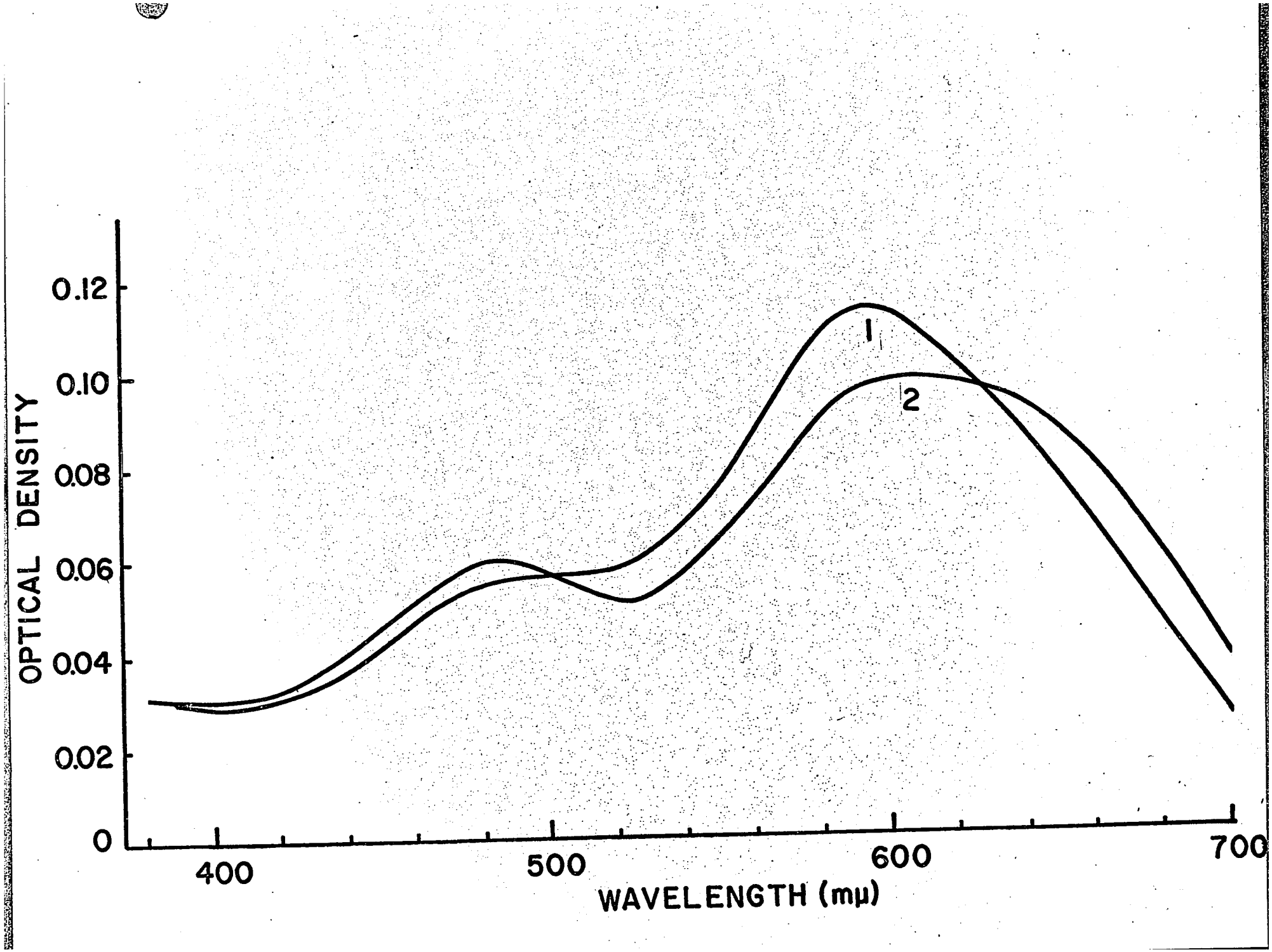
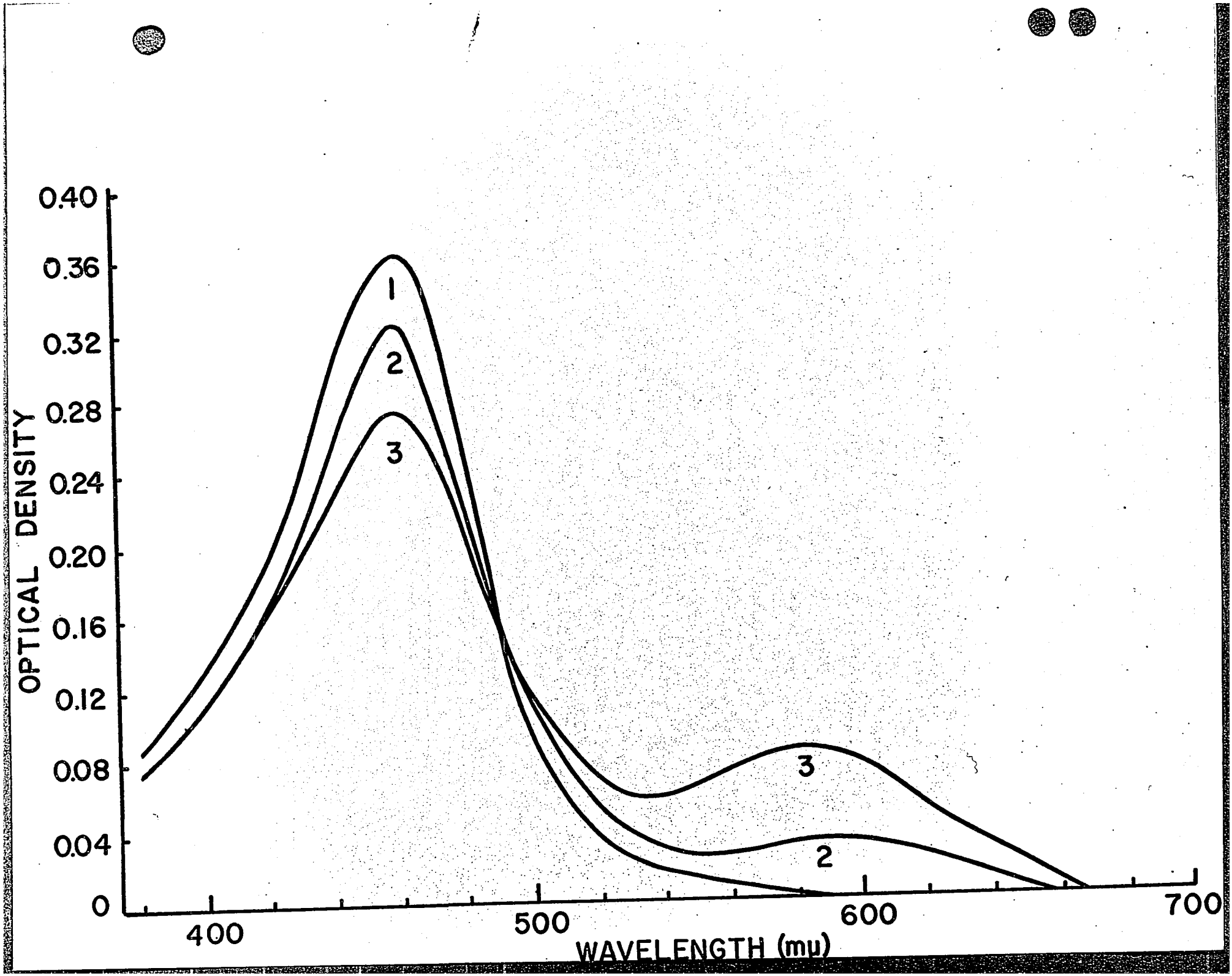

OPTICAL DENSITY
0.12
0.10
0.08
0.06
0.04
0.02
0
400
500
600
700
WAVELENGTH (mμ)
1
2

Figure 31

Effect of chloride ion concentration on the spectrum of a IN-3, 6S-2DNP——BSA solution at pH 4.7. Curve #1 - Buffer containing dye only (μ = 0.02), Curve #2 - Dye plus BSA, μ = 0.1 chloride ion, Curve #3 - Dye plus BSA, μ = 0.02 chloride ion.


0.40
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
OPTICAL DENSITY
1
2
3
3
2
400
500
600
700
WAVELENGTH (mμ)

other investigators to observe a spectral change in this system (166).

Evaluation of Inhibition of Binding by Gel Chromatography

In the above discussions of the effects of various inhibiting substances on the IN-2S-4DNP ——— BSA reaction, it has been tacitly assumed that any reduction of the IN-2S-4DNP spectral shift was due to displacement of this dye from its binding sites on the protein. To test this assumption, chromatographic experiments were performed using IN-2S-4DNP ——— BSA solutions containing sufficient amounts of inhibitor to completely remove the spectral shift of the dye.

The results of experiments of this nature, all at pH 5, μ=0.02, in solutions containing urea, dinitrophenol and 1-Napthol 2-sulfonic acid are shown in Figs. 32 - 34 respectively.

The chromatographic experiment performed in urea at a concentration (6.2 M) that was expected to drastically denature the protein (178) and therefore destroy dye binding sites of "native" BSA, indicated that all of the dye present was free in solution, [Fig. 32]. On the other hand, in solutions containing dinitrophenol (2.6×10^{-3} M, [Fig. 33]) or 1-Napthol 2-sulfonic acid (3.7×10^{-3} M, [Fig. 34]) it appeared as if most of the dye present was still bound to the protein; even though no spectral shift could be seen. However, a comparison of the last two figures reveals that a marked tailing of the IN-2S-4DNP absorption occurred in the presence of dinitrophenol. As determination of the protein absorbance (by the Folin-Ciocalteu method) indicated that its elution pattern was in the form of a symmetrical peak, it would seem that this tailing was due to a reduction in the affinity of IN-2S-4DNP for BSA in the presence of dinitrophenol.

Figure 32

Chromatogram of a 1N-2S-4DNP———BSA solution in 6.2 M urea. Curve #1 - Absorption at 280 mμ, Curve #2 - Absorption at 480 mμ.

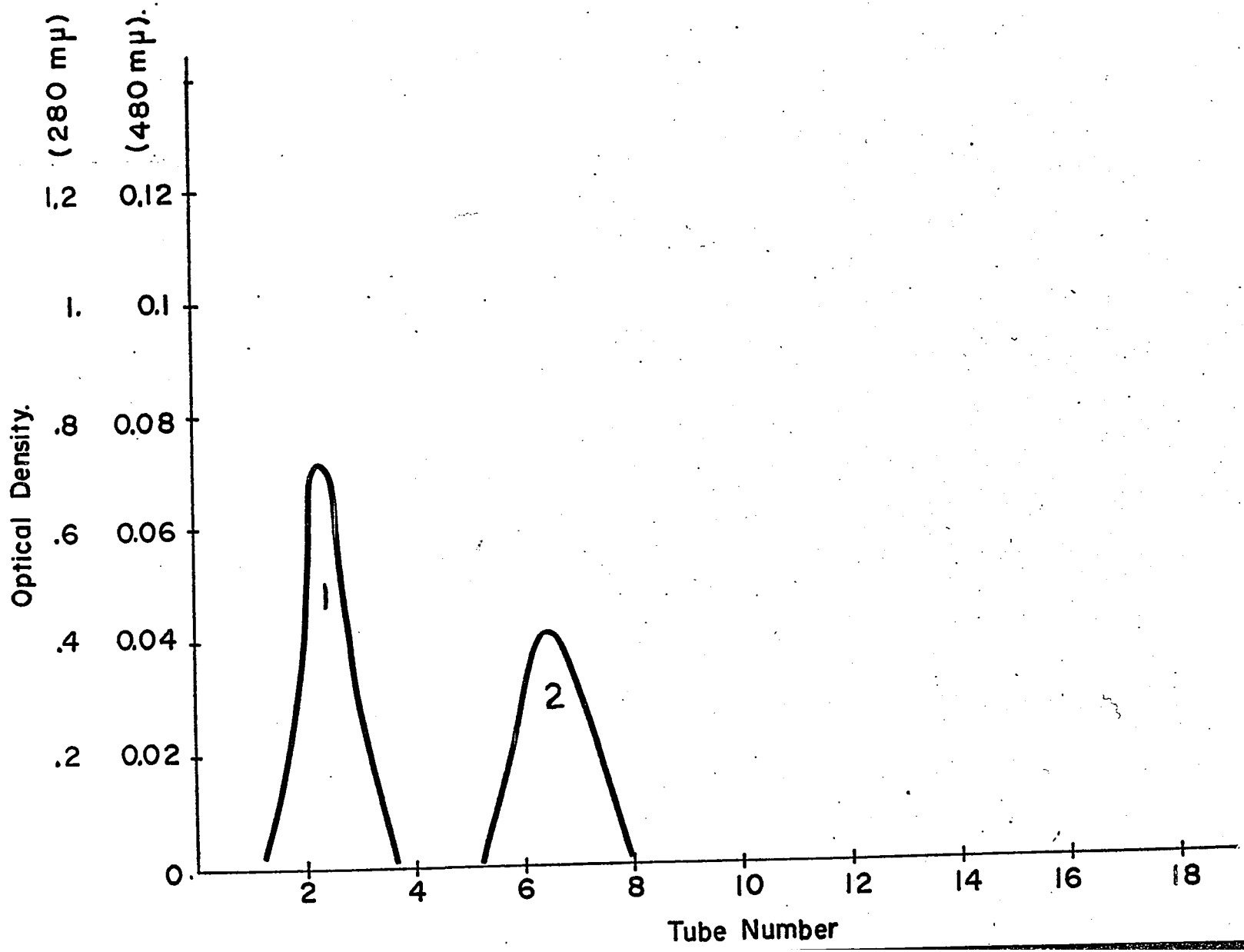

Optical Density.
(280 mμ)
(480 mμ).
1.2
0.12
1.
0.1
.8
0.08
.6
0.06
.4
0.04
.2
0.02
0.
1
2
2
4
6
8
10
12
14
16
18
Tube Number

Figure 33

Chromatogram of a 1N-2S-4DNP——BSA solution containing 2.5×10^{-3} M 2,4-Dinitrophenol at pH 5. Curve #1 - Absorbance (at 600 mμ) of aliquots of chromatographed Dye-BSA, dinitrophenol solution containing Folin Ciocalteu reagent, Curve #2 - Absorbance (at 600 mμ) [after addition of 1N-NaOH] of the 1N-2S-4DNP——BSA - 2, 4-dinitrophenol solution. Curve #3 - Absorbance (at 480 mμ) of a 1N-2S-4DNP——BSA solution, Curve #4 - Absorbance (at 600 mμ after addition of 1N-NaOH) of a 1N-2S-4DNP - 2, 4-dinitrophenol solution

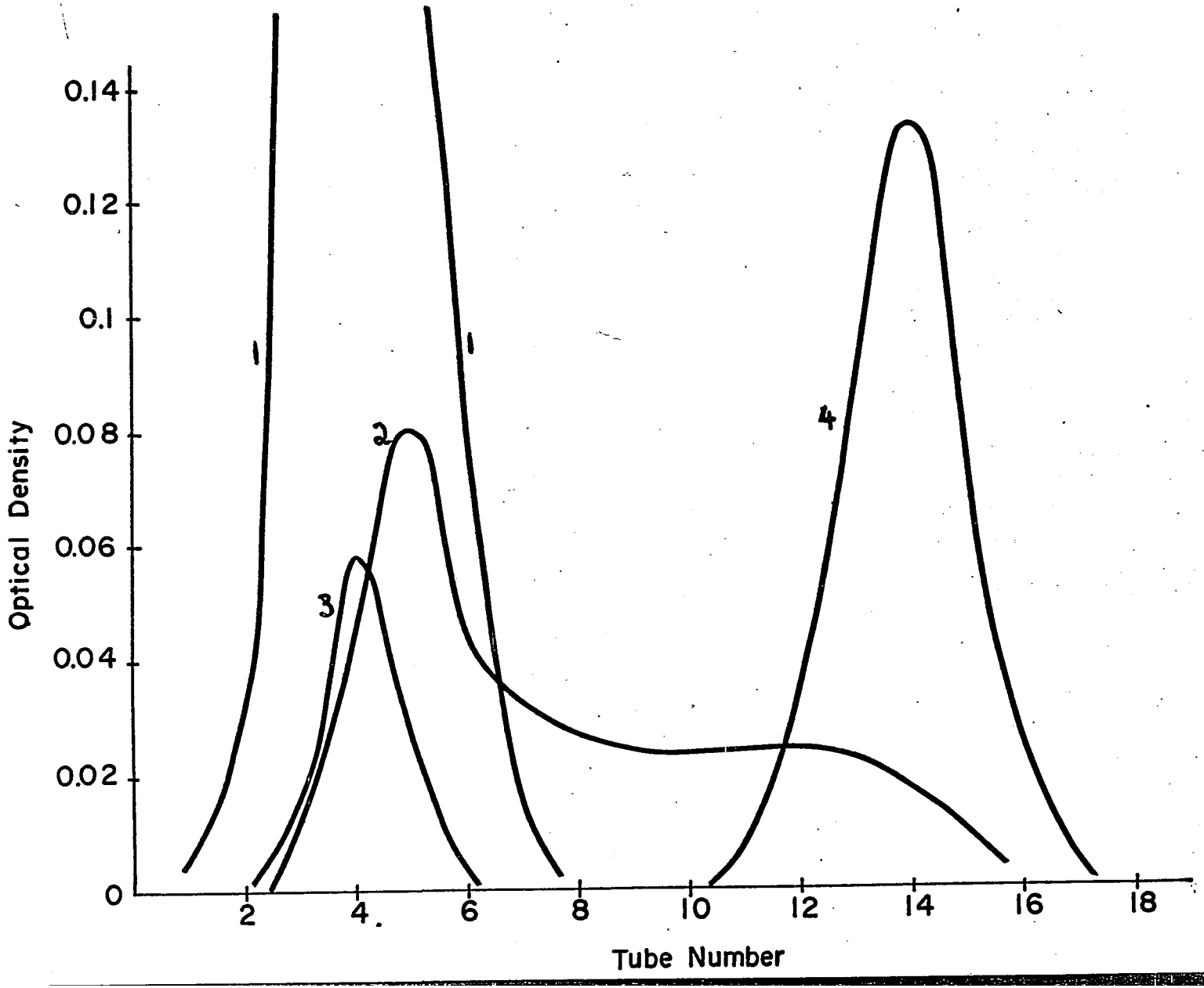
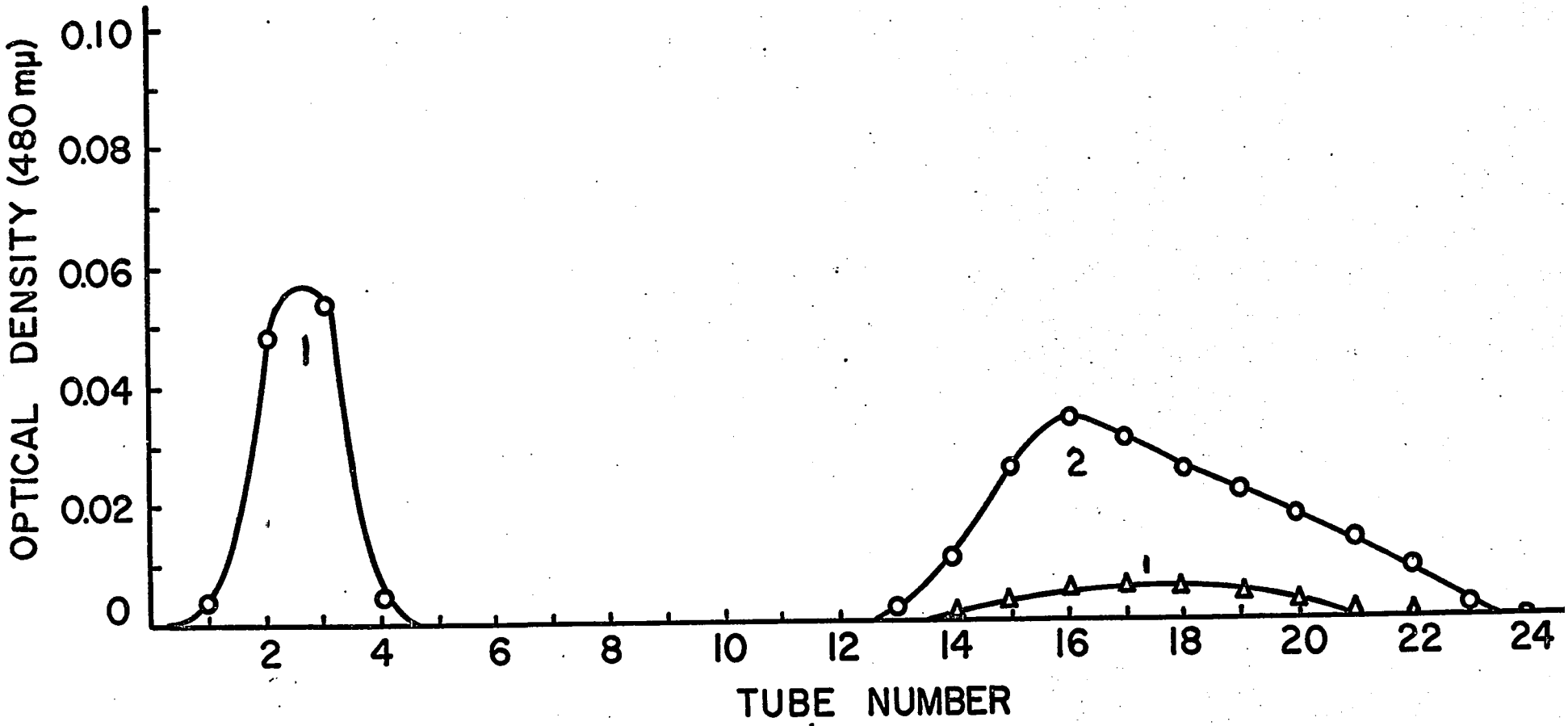

0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Optical Density
1
1
2
3
4
2
4
6
8
10
12
14
16
18
Tube Number

Figure 34

Chromatogram of a IN-2S-4DNP————BSA solution containing 3×10^{-2} M 1-Napthol - 2-sulfonic acid. Curve #1 - Absorbance in the presence of BSA. Curve #2 - Absorbance of IN-2S-4DNP plus 1-Napthol, - 2-sulfonic acid only.


OPTICAL DENSITY (480 mμ)
0.10
0.08
0.06
0.04
0.02
0
2
4
6
8
10
12
14
16
18
20
22
24
TUBE NUMBER
1
2
1'

The results of these chromatographic experiments therefore indicate that different effects are involved in the inhibition of the spectral shift by urea and by the napthol and nitrophenol derivatives. The effect of the latter set of compounds can be interpreted in either of two ways: I) they cause an apparent increase in the pK of the bound dye in a similar fashion to that caused by thiocyanate or nitrate ions, or (II) they have some effect, either through competition or by causing a conformational change on a group of the protein which interacts with the bound dye to cause the spectral shift. A clear cut choice between these alternatives cannot be made on the basis of the data presented here, although a combination of both of these mechanisms might be needed to describe the observed phenomena (for further discussion of this please see the next section).

An analysis of the comparative effects of the mononitrophenols and dinitrophenol on the spectral shift of IN-2S-4DNP would seem to indicate that by analogy the much greater inihibitory efficiency of dinitrophenol might be traced to its pK of 3.96 (compared to 7.16 for the mononitrophenols) as a result of which it is almost completely ionized at pH 5. Even if the mononitrophenols were as strongly bound as dinitrophenol to the protein, the fact that they are uncharged at this pH would seem to prevent them from affecting the spectral shift.

The differences between the effects of the three napthol sulfonic acids and 1-napthol were not as clear-cut as the results obtained with the various nitrophenols tested. As in the case of the latter, however, it is probable that the reduction of the spectral shift caused

by these reagents was not due to a displacement of 1N-2S-4DNP from the protein, but rather to a non-specific effect on the titration curve of the bound dye. As both the 1, 2- and 1, 4-napthol sulfonic acids were equally effective, their mode of action would not seem to involve a specific, sterically controlled interaction of the naptholic hydroxyl and sulfonic acid groups with the protein. With this in mind, the relative effects of 1-napthol and of the three napthol sulfonic acids appear anomalous.

Thus on the basis of the assumption that hydrophobic bonds are the principle stabilizers of protein-organic molecule interactions (166, 171) the order of their affinity for the protein would be expected to be 1-napthol, 1-napthol 2- or 4-sulfonic acid, 1-napthol, 3, 6-disulfonic acid. On the other hand, any increase in proton binding by the protein produced by these substances (if they had equal affinities for the protein) would be expected to be in the reverse order, because of their increasing negative charge. If these assumptions are correct it would appear that the effect on the spectral shift depends on a combination of two parameters i.e. "hydrophobic affinity" and charge, such that the charged napthol monosulfonic acids are more effective than 1-napthol because of their greater charge and also more effective than the napthol disulfonic acid because of the reduced affinity of the latter for the protein.

GENERAL DISCUSSION

The interaction of 1N-2S-4DNP with BSA has been shown to result in an unusually striking spectral shift, similar to that previously observed in the reactions of (a few) other azo-dye pH indicators both with this protein, and with specific antibodies (121, 166-169). These molecules thus

appear to have certain common characteristics which enable them to easily undergo large apparent pK shifts when bound to proteins. It is difficult, however, to make direct comparisons of the reactions of the systems studied to date, not only because of the different structures, and determinant groups of the dyes used, but also because so little is known of the actual mechanisms involved in the interactions between a bound (dye) molecule and the appropriate groups of the protein binding site(s). The interaction of 1N-2S-4DNP with BSA has been investigated in some detail in the hope that a greater understanding of these reactions could be obtained.

A general consideration of the effect of the various inhibitors tested on the dye-protein interaction leads to the conclusion that the binding of 1N-2S-4DNP results from the formation of unique dye-protein bonds. This is supported by the observation that the dye was not displaced from its binding site by related molecules such as 1N-2S-4pNP (or 1N-3, 6S-2DNP) or even by a 10,000-fold excess of 1-napthol 2-sulfonic acid. The fact that 1N-2S-4pNP, which has the same pK as 1N-2S-4DNP and differs from it only in its lack of a nitro group, exhibited completely different behavior from the latter, also is indicative of the importance of the contribution of all parts of the molecule, in this case of the 2-nitro group, to the final, overall dye protein bond formed. As was also noted, the dye 1N-2S-4DNP was firmly bound to the protein under conditions where no spectral shift was observed, (i.e. at pH 2). Thus, it is obvious that, as has been found in other systems (156), no direct correlation can be made between the spectral shift of a given dye-protein system and the nature and strength of the dye-protein bond formed.

The pH dependence of the IN-2S-4DNP —— BSA spectral shift is similar in some respects to the pH dependence observed in the binding of other compounds, e.g. skatole and acetyl-L-tryptophan (179) by normal BSA and crystal violet (180) by the denatured protein.* Four plausible explanations, considered below, may account for the titration behavior at low pH of IN-2S-4DNP bound to BSA:

1) The possibility that the N-F transition of BSA has some effect on the spectral shift is not likely as the former occurs in the pH range of 3.5 to 4.5 (123). This does not correspond to that observed in the titration of bound IN-2S-4DNP.

2) Klotz et al (181) have found that the pK of an azo mercurial dye i.e. 4-(p-dimethylaminobenzeneazo)-phenyl mercuric acetate, shifted from 3.3 in water, when it was in the form of a cysteine complex, to about 1.8 when this dye was coupled to the SH group of mercaptalbumin. This phenomenon was interpreted by postulating that an envelope of structured water molecules ("icebergs") surrounded hydrophobic regions of the protein, as a result of which the titration behavior of the coupled dye differed from that of the cysteine complex free in solution. As a similar effect on the pK has been noted in the BSA—— IN-2S-4DNP system (even though this dye was not chemically coupled to the protein), a comparison with the foregoing system might lead to the conclusion that a similar explanation would also hold in this case. However, one important

* In these last two cases the dependence of binding on pH was respectively identified with the occurrence of the N-F transition (179) and with protonation of carboxylate groups resulting in a decrease in binding sites (180).

difference in the properties of the two systems should be noted. Klotz et al (181) observed that the addition of sodium dodecyl sulfate, (S.D.S.) sufficient to increase the negative charge of the protein by approximately 30 charge units, caused an even further decrease in the pK of the bound azo mercurial dye. Also, the pK of the coupled dye, calculated at different points of its titration curve, did not decrease with a decrease in pH, while the titration curve itself could not be fitted to the standard equation $pK = pH - \log \frac{[A]}{[AH]}$ where [A] and [AH] are the concentrations of the neutral and protonated forms of the dye respectively. These results were interpreted as indicating that the coupled dye did not behave in accordance with general electrostatic theory and were, therefore, taken as supporting evidence for the "iceberg" hypothesis. As has been shown in the case of IN-2S-4DNP, however, the effect of anions on the titration curve of the bound dye was as expected from a prediction of the electrostatic effects of bound anions on the titration properties of a protein. On the basis of this difference in properties of the two systems it would appear, therefore, that the "iceberg" hypothesis would not provide a suitable explanation for the pK shift of IN-2S-4DNP. It should also be pointed out that the interpretation of the reasons for the pK shift of the mercurial dye does involve certain difficulties. Thus, it has been pointed out that, under the conditions of the titration of the coupled mercurial, BSA is wholly or partly unfolded (123). Furthermore,

no attempt was made to distinguish between the effects of bound S.D.S. molecules on the protein's charge and any possible effect on the protein's structure or conformation, due to the considerable alyphatic character of S.D.S. Indeed at higher S.D.S. concentrations the pK of the coupled mercurial dye was found to shift to a higher pH than that of the free form. This effect was ascribed to the formation of S.D.S. micelles on the protein's surface, since a similar pK shift was noted when the free dye (in the form of a cysteine complex) was titrated in solutions containing micelles of this detergent. In view of this complication it would appear that conclusions drawn on the basis of the effects of simpler anions would be more unequivocal.

3) It is possible that the binding of IN-2S-4DNP to BSA allows an interplay of forces that alters the electronic structure of the dye. For example hydrogen bonding between groups on the protein and nitro groups of the dye could induce a reduction in the electronic density at the oxygen of the napthol ic OH group with a concomitant decrease.in the pK of the dye. In this case the titration curve observed would be that of the napthol ic OH group itself.

4) As an alternate to the last explanation (#3), binding of the dye (IN-2S-4DNP) to the protein could be considered to allow the formation of a strong hydrogen bond (or actual proton transfer) between the napthol ic OH group and carboxylate groups of the protein. In this case titration

of the protein at low pH would reduce the concentration of carboxylate ions available for the formation of this bond and thus lead to a reduction of the spectral shift. The fact that the titration of the bound dye occurred in the same pH region as that of carboxylate groups of BSA* and that the effect of thiocyanate (and of the other amonic compounds tested) on the titration of the bound dye was similar to the effect of anions on the protein's titration (23) can be taken as supporting evidence for this postulate. However, it is also possible that the correspondence of these two titrations with respect to pH is purely fortuitous and that the effect of anions is nonspecific, reflecting the general increase in proton binding by the protein at a given pH.

The purpose of these experiments was to obtain a greater understanding of the reactions of dyes with proteins that lead to spectral shifts. This was achieved to a certain extent, however, no exact, unequivocal description could be made of the interactions leading to the observed spectral changes. In retrospect, it appears that the approaches used in this study may have been inherently incapable of providing answers to these problems, since it is difficult, if not possible, to differentiate between the effects of added reagents on the behavior of the bound dye and on the properties of the protein itself. Such complexities are to be expected in systems consisting of complex biopolymers such as BSA,

* Exact correspondence of these titrations should not be expected as there are approximately 90 carboxylate groups per BSA molecule (182) while the number of IN-2S-4DNP protein binding sites per molecule was calculated as 4 to 5.

and might be overcome by the use of other techniques such as nuclear magnetic and electron spin resonance or infrared microscopy with which one might be able to "probe" the ligand-protein complex without interfering with the reaction itself.

CHAPTER III

The Interaction of Anti-Dinitrophenyl Rabbit Antibodies with Two Azo-Dye Haptens Containing the 2, 4-Dinitrophenyl Determinant Group

PART A

A Comparison of Spectrophotemetric and Equilibrium Dialysis Techniques for the Evaluation of Antibody-Hapten Interactions

INTRODUCTION

Many compounds of low molecular weight have been found to exhibit spectral changes in the presence of proteins. For example, the study reported in Chapter II of this thesis can be taken as representative of one of the more striking spectral changes encountered in an albumin azo-dye system. Similarly, changes have been observed in antibody-dye systems (96, 104) and in some non azo-dye hapten-antibody systems (64). As has been stated, the underlying causes of these spectral changes are not well understood. In general, however, they can be considered to reflect on alteration in the environment of a small molecule on binding to a protein and more specifically, to be due, in certain cases, to factors such as electronic interactions (64, 153) and/or conformational changes (153) in the small molecule itself.

The extent of binding in protein-dye systems can be calculated from measurements of the extent of a spectral change using a method developed by Klotz (82, 83). This procedure has been used in Chapter II of this thesis and a similar method has been employed in order to determine the extent of combination of antibodies with haptens (120, 121, 166). According to this method the fraction of dye molecules present in the bound form, β, can be calculated by use of equation [1. Ch. I]

$$\beta = \frac{\epsilon app - \epsilon f}{\epsilon b - \epsilon f} \qquad [1]$$

where ϵ_f, ϵ_b are extinction coefficients for the free and bound forms of the dye molecule and ϵ_{app} is the overall extinction coefficient for the dye-protein solution, all at the same wavelength.

A basic assumption inherent in the use of the above equation is that ϵ_b is the same for every bound dye molecule. However, in view of the known heterogeneity of antibodies with respect to their binding constants, it is conceivable that ϵ_b will vary and depend on the nature of the interactions with the particular antibody with which the dye combines. As the spectrophotometric method was to be used in the present study it was felt that it was necessary to test this assumption by comparing the results of spectrophotometric binding measurements in a dye-hapten (1-napthol 2-(2, 4-dinitrophenylazo) 3, 6-disulfonic acid [1N-3, 6S-2DNP]) antibody system with the absolute results of an unequivocal technique, equilibrium dialysis. The interaction of this dye-hapten with antidinitrophenyl antibodies has been previously described by Metzger et al (166).

MATERIALS

Hapten

The dye IN-3, 6S-2DNP - was obtained from Eastman Chemical Co., Rochester, New York. The elemental analysis supplied by Eastman for this compound and its theoretical composition are listed below:

	C %	H %	N %	S %
Calculated for $C_{16} H_8 O_{11} N_4 S_2 Na_2 .3H_2O$	35.4	1.5	10.3	11.8
Found	35.6	1.3	10.4	12.0

Antibodies

The production of antibodies to the 2, 4-dinitrophenyl (DNP) group was elicited in rabbits by the injection of a DNP-bovine gamma globulin conjugate. The bovine protein was obtained as Fraction II from Armour Pharmaceutical Co., Kankakee, Illinois, and the antigen was prepared according to the coupling procedure of Eisen et al (184, 66). An approximately 1000 molar excess of 2, 4-dinitrobenze sulfonic acid was used in the coupling procedure so that in the resultant protein conjugate all lysine ϵ-amino groups could be considered to have reacted with this reagent. Each rabbit was given injections of 0.4 mg of protein conjugate in 0.4 ml. of Freunds adjuvant into each of its footpads simultaneously. The animals were bled about 6 weeks after the initial injection and at monthly intervals thereafter. Intramuscular booster injections were also administered after each bleeding to improve the antibody titer.

The globulin fractions of pools of sera obtained from single rabbits were isolated by sodium sulfate precipitation (36); "normal"

rabbit gamma globulin (NRG) preparations were obtained in the same way from non-immunized rabbits.

Binding Measurements

All experiments were carried out in borate buffer, pH 8.0, μ= 0.15, Antibody and NRG solutions used for spectral and equilibrium dialysis experiments were 2.5×10^{-4} M in total protein.

Spectra were measured at 24-26°C in a Unicam SP 500 spectrophotometer using microcells of 1 cm path length and 0.5 ml capacity. Where necessary, protein solutions of appropriate concentration were used as blanks.

The extinction coefficient of the bound hapten, ϵ_b, was determined by addition of the dye hapten in increasing concentrations to a constant amount of antibody; the ratio of antibody to hapten concentrations was maintained at a relatively high value so that the contribution of free hapten could be neglected. In experiments designed for the determination of binding constants, hapten solutions of higher concentration were added to solutions of the same antibody preparation. The values of ϵ_{app} obtained from the latter experiments were used to calculate the amount of hapten bound as a function of free hapten by means of equation [1].

Equilibrium dialysis was carried out at 25°C in glass cells containing 1 ml of protein solution separated from 1 ml of hapten solution by a Visking dialysis membrane, treated to remove soluble impurities according to the procedure of Hughes and Klotz (185). The dialysis cells were constructed of two half cells, each of two ml total capacity, flanged at one end and closed at the other by a plastic screw cap lined with parafilm. The dialysis membrane was clamped between the flanges of the

two half cells and the joint formed covered with sealing wax to prevent leakage. Equilibration was achieved by rotation of the cells for 24 hours. To measure the degree of non-specific binding, control experiments were performed with appropriate NRG soltuions.

The average equilibrium constant K_o and the concentration of antibody sites [Ab], were evaluated from the spectrophotometric and equilibrium dialysis binding data by the use of equation [3],*

$$\frac{1}{b} = \frac{1}{K_o c[Ab]} + \frac{1}{[Ab]} \qquad [3]$$

where b and c are the concentrations of bound and free hapten, respectively (91).

RESULTS

Properties of the Free Dye

The dye IN-3, 6S-2DNP is a pH indicator changing from yellow in acid to blue in basic solution [Fig. 35], with an apparent pK, evaluated spectrophometrically at 590 mμ, of 6.52 (166). The dye was found to obey Beer's law at all concentrations used at pH 8, i.e. up to 5×10^{-5} M, and its solutions were stable at room temperature for as long as 3 months. The extinction coefficient at 590 mμ at pH 8, the absorption maximum of the free dye at this pH, was calculated as 4.11×10^4 M^{-1} cm^{-1}.

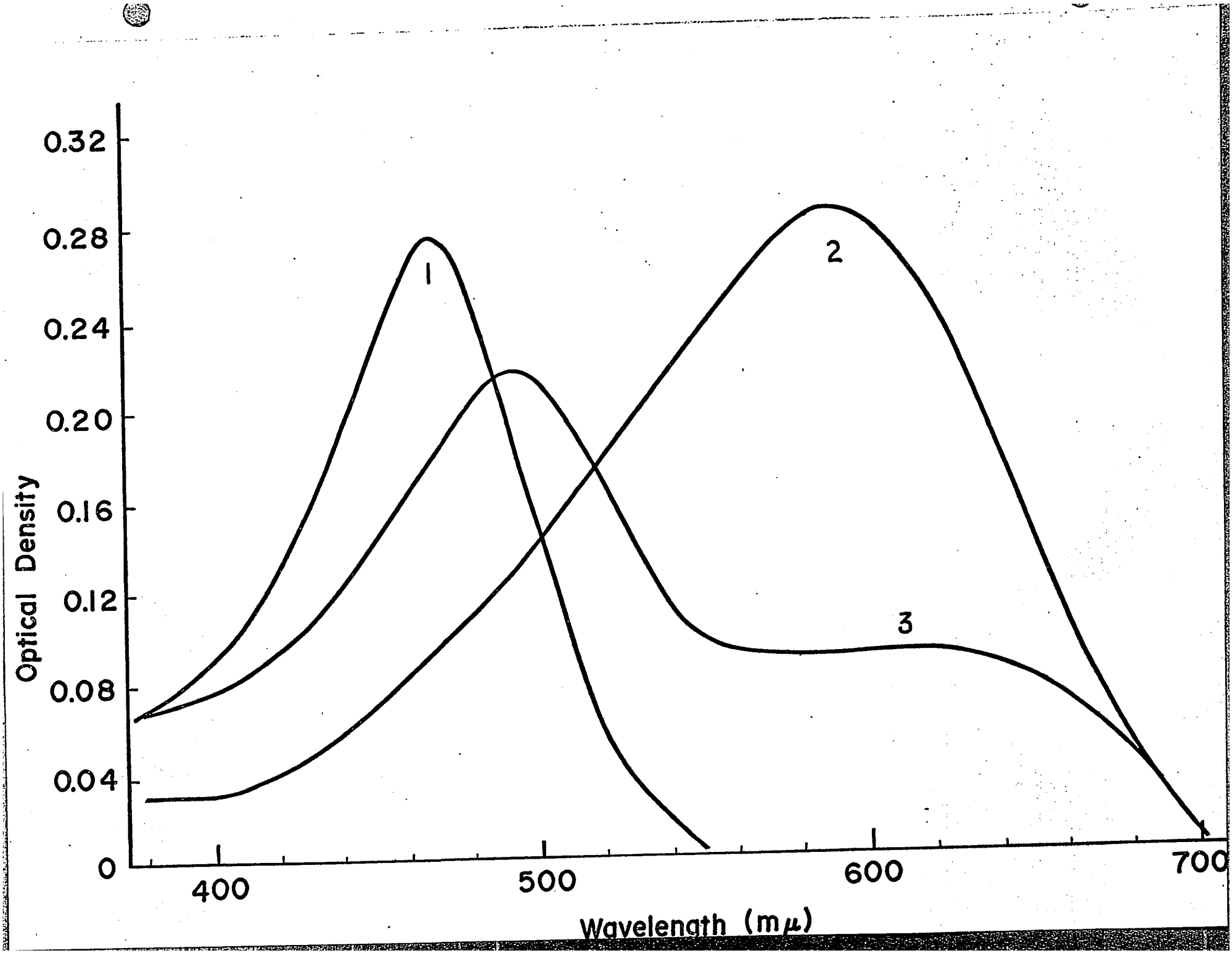
Hapten-Antibody Interactions

The effects of an anti-DNP-antibody preparation (8×10^{-5} M) and of an NRG preparation of the same total protein concentration (8.5×10^{-4} M)

* This equation is equivalent to equation [3] of Ch. I.

Figure 35

Spectrum of a 6.8×10^{-6} M IN-3, 6S-2DNP solution under different conditions. Curve #1 - Buffer pH 4.5, $\mu = 0.15$, Curve #2 - Buffer alone or buffer containing 8.5×10^{-4} M NRG, pH 8, $\mu = 0.15$, Curve #3 - Buffer plus anti-DNP antibody (8×10^{-5} M) pH 8, $\mu = 0.15$.


0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
Optical Density
1
2
3
400
500
600
700
Wavelength (mμ)

on the spectrum of IN-3, 6S-2DNP at pH 8, $\mu = 0.15$ are shown in [Fig. 35]. As can be seen the NRG preparation had no effect on the dye's spectrum; on the other hand binding to antibody caused a shift of its spectrum to shorter wavelengths, to the range corresponding to the absorbance of the free dye at low pH.

In spite of the fact that no spectral change was observed in the presence of the NRG preparation, equilibrium dialysis experiments revealed that 9% of the dye, in the concentration range of 1×10^{-6} - 12×10^{-6} M, was bound non-specifically by the NRG solution, [Fig. 36]. Control experiments, using protein free buffer solutions, indicated that in the same concentration range 12.5% of the free dye was absorbed to the dialysis membrane [Fig. 36]. Appropriate corrections for both these effects were made in calculating results of spectrophotometric and equilibrium dialysis experiments.

The evaluation of the bound extinction coefficient, ϵ_b, at 590 mμ is shown in Fig. 37. The value obtained for ϵ_b, 0.98×10^4 M^{-1} cm^{-1}, was used to calculate the amount of hapten bound in antibody solutions of relatively higher total hapten concentration. The results of such spectrophotometric and equilibrium dialysis experiments, performed with solutions of the same antibody and protein concentration, are given in Tables V and VI respectively. The concentration of hapten bound, b, for both these experiments, is plotted as a function of the sum of bound and free, b and c, hapten concentrations in Fig. 38. The data are also represented in the form of a $1/b$ vs $1/c$ plot, in accordance with equation [3] in Fig. 39; the curvature of the plots being an obvious indication of the heterogeneity of the system. The values of k_o and [Ab], calculated from this figure, for equilibrium dialysis and spectrophotometric

Figure 36

Concentration of IN-3, 6S-2DNP bound by NRG preparation and dialysis membrane (0-0-0) or by dialysis membrane alone •-•-• as a function of free dye concentration.

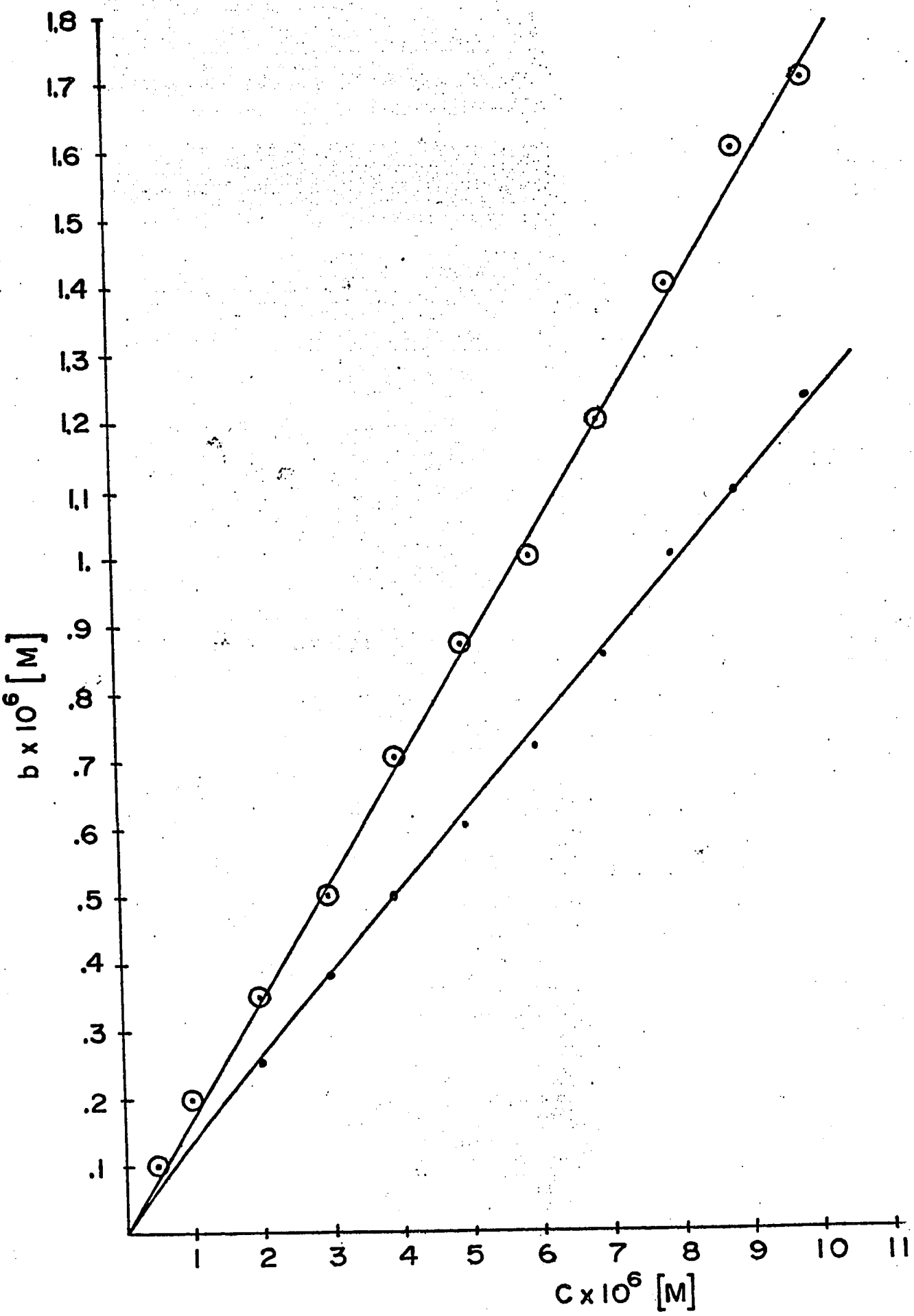

1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.
.9
.8
.7
.6
.5
.4
.3
.2
.1
b x 10^6 [M]
1
2
3
4
5
6
7
8
9
10
11
C x 10^6 [M]

Figure 37

Evaluation of ϵ_b for IN-3, 6S-2DNP bound to anti-DNP antibody at pH 8, μ= 0.15.

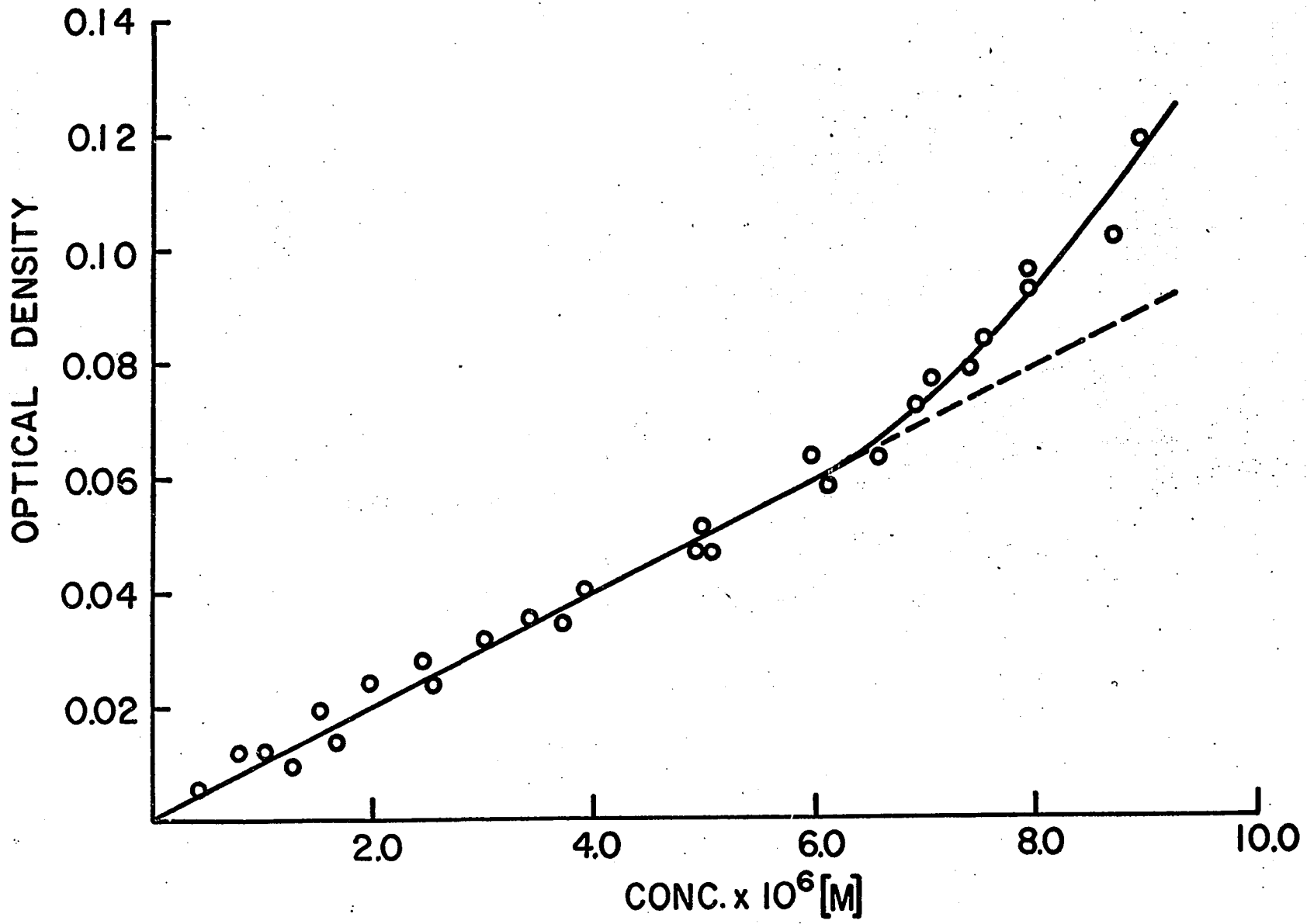

0.14
0.12
0.10
0.08
0.06
0.04
0.02
OPTICAL DENSITY
2.0
4.0
6.0
8.0
10.0
CONC. x 10⁶ [M]

Table V

Binding of IN-3, 6S-2DNP by Anti-DNP Antibodies

Spectrophotometric Results

IN-3, 6S-2DNP added $\times 10^6$ [M]	O.D. 590 mμ	ϵ_{app} $\times 10^{-4}$ [$M^{-1}cm^{-1}$]	β	b $\times 10^6$ [M]	c_{total} $\times 10^6$ [M]	c cor. (× 90.9%) $\times 10^6$ [M]	b+c	$1/_b$ $\times 10^{-6}$ [M^{-1}]	$1/_c$ $\times 10^{-6}$[M^{-1}]
4.99	0.051	1.021	0.987	4.92	0.07	0.064	4.98	0.203	1516
5.97	0.063	1.055	0.975	5.81	0.16	0.15	5.96	0.172	6.66
7.06	0.079	1.13	0.955	6.74	0.32	0.29	7.03	0.145	3.45
7.40	0.088	1.19	0.932	6.90	0.50	0.45	7.35	0.145	2.20
7.91	0.092	1.16	0.942	7.45	0.46	0.42	7.87	0.134	2.38
7.93	0.095	1.20	0.930	7.37	0.56	0.51	7.88	0.136	1.96
8.70	0.101	1.16	0.942	8.20	0.50	0.45	8.65	0.122	2.22
8.94	0.118	1.32	0.891	7.94	0.97	0.88	8.85	0.126	1.14
9.93	0.136	1.37	0.875	8.69	1.24	1.13	9.82	0.115	0.886
10.2	0.141	1.38	0.872	8.89	1.31	1.19	10.08	0.113	0.840
11.1	0.160	1.44	0.853	9.47	1.63	1.48	10.95	0.106	0.676
12.4	0.194	1.57	0.812	10.08	2.32	2.11	12.19	0.0995	0.475
13.45	0.224	1.67	0.779	10.48	2.97	2.68	13.16	0.0955	0.374

Table V (Continued)

IN-3, 6S-2DNP added $\times 10^6$ [M]	O.D. 590 mμ	ϵ_{app} $\times 10^{-4}$ [$M^{-1}cm^{-1}$]	β	b $\times 10^6$ [M]	c total $\times 10^6$ [M]	c cor. (x 90.9%) $\times 10^6$ [M]	b+c	$1/_b$ $\times 10^{-6}$ [M^{-1}]	$1/_c$ $\times 10^{-6}$ [M^{-1}]
14.0	0.257	1.76	0.750	10.95	3.05	2.77	13.72	0.915	0.362
15.0	0.285	1.90	0.706	10.59	4.41	4.01	14.60	0.945	0.250
15.3	0.282	1.84	0.725	11.09	4.29	3.90	14.99	0.904	0.256
15.83	0.301	1.90	0.705	11.19	4.66	4.24	15.43	0.895	0.236
17.6	0.369	2.10	0.642	11.30	6.30	5.71	17.01	0.885	0.175
18.2	0.383	2.12	0.635	11.56	6.64	6.20	17.76	0.865	0.161

Table VI

Binding of IN-3, 6S-2DNP by Anti-DNP Antibodies

Equilibrium Dialysis Results

IN-3, 6S-2DNP added $\times 10^6$ [M]	c buffer side $\times 10^6$ [M]	b non-specific $\times 10^6$ [M]	b $\times 10^6$ [M]	$1/_b$ $\times 10^{-6}$ [M^{-1}]	$1/_c$ $\times 10^{-6}$ [M^{-1}]
3.94	0.18	0.07	3.50	0.286	5.55
5.98	0.33	0.13	5.19	0.193	3.15
6.04	0.39	0.15	5.11	0.196	2.56
6.18	0.46	0.17	5.10	0.196	2.18
8.20	0.78	0.28	6.36	0.157	1.28
8.22	0.83	0.30	6.26	0.160	1.21
8.30	0.61	0.23	6.85	0.146	1.64
10.12	1.22	0.43	7.28	0.137	0.820
10.18	1.24	0.44	7.26	0.138	0.806
10.68	1.31	0.46	7.60	0.132	0.765
12.30	1.70	0.59	8.31	0.120	0.589
12.56	1.87	0.65	8.17	0.123	0.535
14.56	2.53	0.88	8.62	0.116	0.396

Table VI (Continued)

Binding of IN-3, 6S-2DNP by Anti-DNP Antibodies

Equilibrium Dialysis Results

IN-3, 6S-2DNP added $\times 10^6$ [M]	c buffer side $\times 10^6$ [M]	b non-specific $\times 10^6$ [M]	b $\times 10^6$ [M]	$1/_b$ $\times 10^{-6}$ [M^{-1}]	$1/_c$ $\times 10^{-6}$ [M^{-1}]
16.24	3.16	1.09	8.83	0.113	0.317
16.60	3.31	1.15	8.83	0.113	0.302
18.44	3.77	1.30	9.60	0.104	0.266
18.88	4.14	1.43	9.17	0.109	0.242
21.20	4.86	1.67	9.81	0.102	0.206

Figure 38

Concentration of IN-3, 6S-2DNP bound by antibody at pH 8, μ = 0.15 as a function of bound and free dye present; -O-O- spectrophotometry, -□-□- equilibrium dialysis.

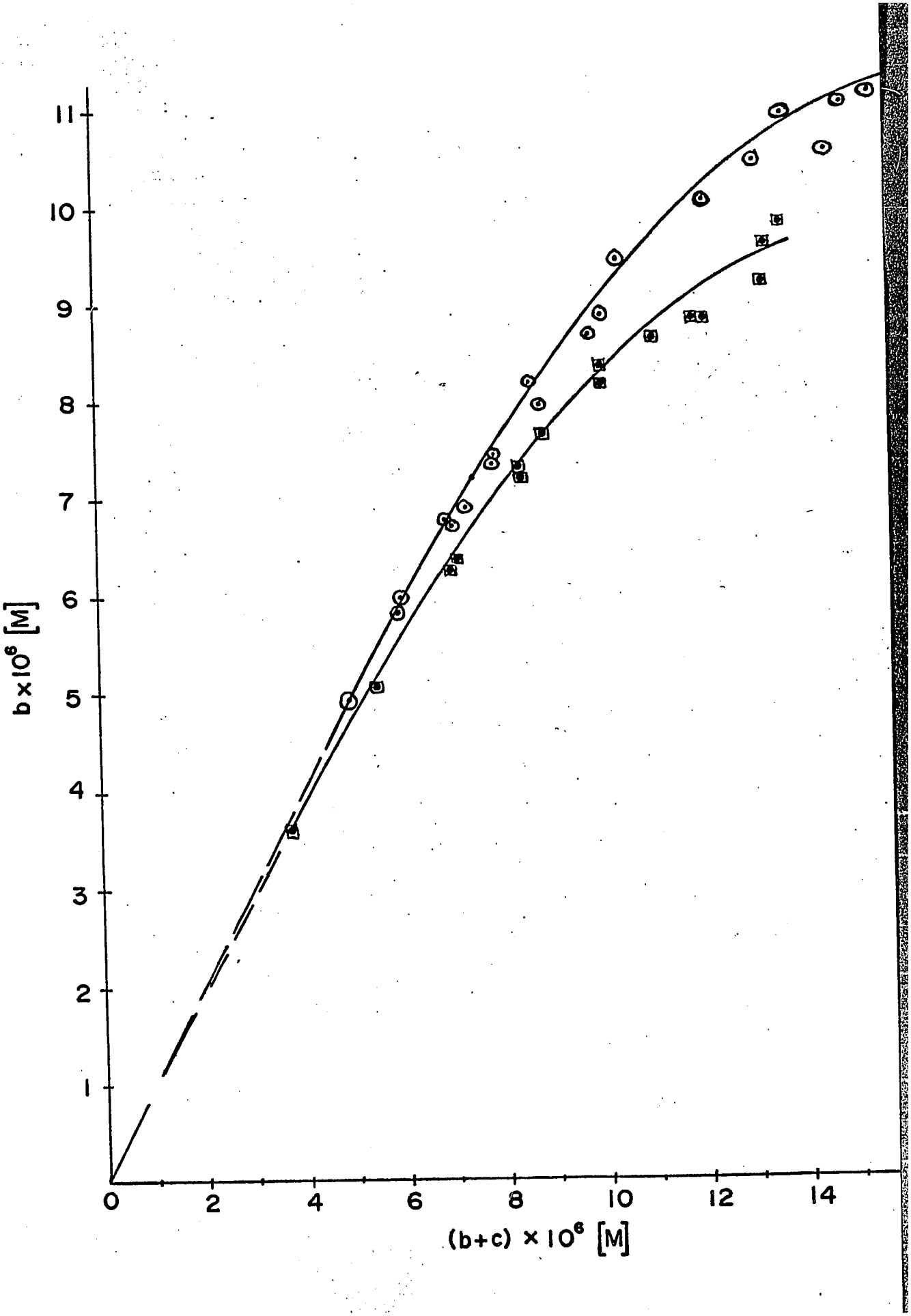
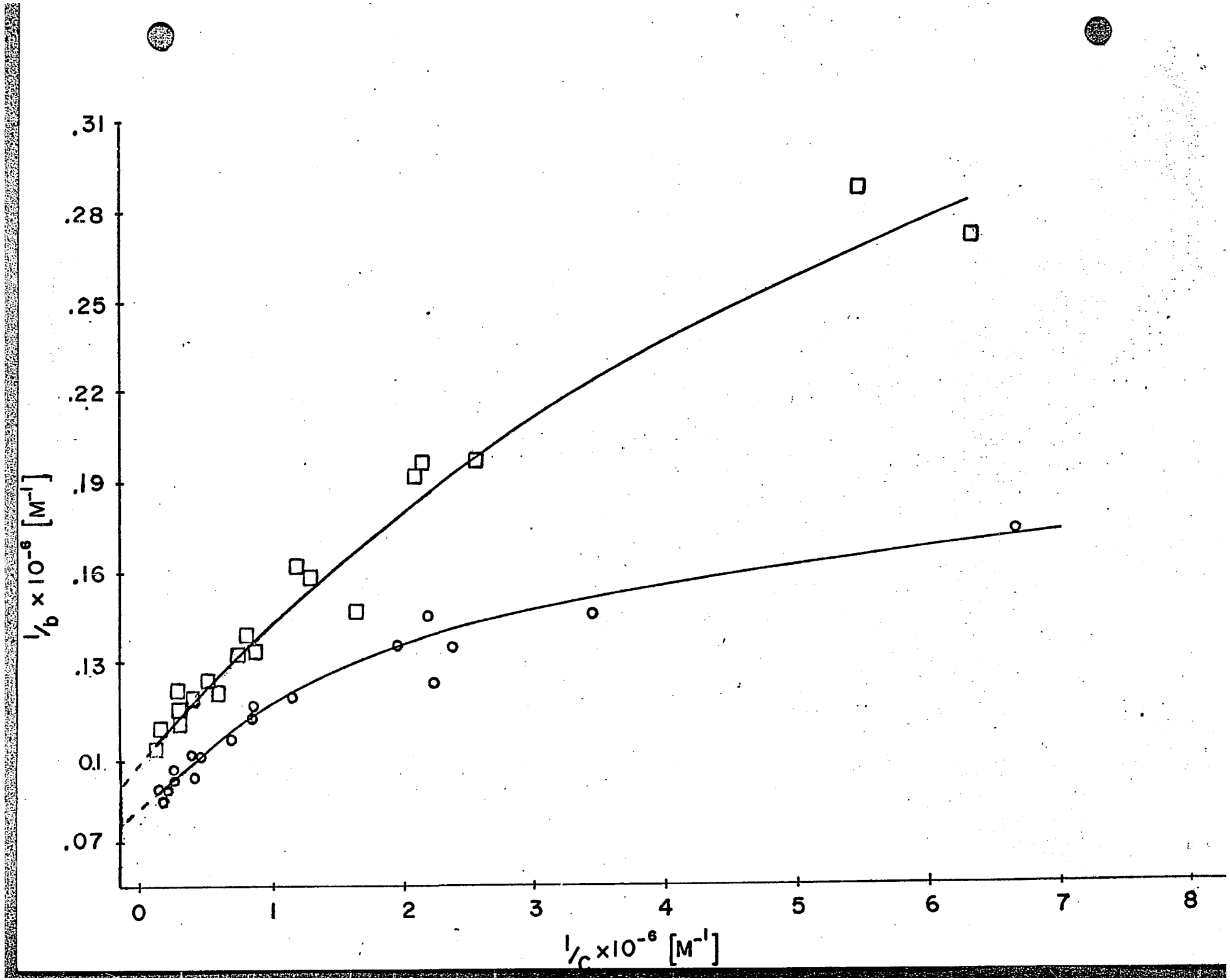

b x 10⁶ [M]
(b+c) x 10⁶ [M]
0
2
4
6
8
10
12
14
1
2
3
4
5
6
7
8
9
10
11

Figure 39

Binding data for the IN-3, 6S-2DNP anti-DNP system at pH 8, μ= 0.15: -O-O- spectrophotometry, -□-□- equilibrium dialysis.


.31
.28
.25
.22
.19
.16
.13
0.1
.07
1/b x 10^-6 [M^-1]
0
1
2
3
4
5
6
7
8
1/c x 10^-6 [M^-1]

experiments are listed below;

	Equilibrium Dialysis	Spectrophotometry
$K_o \times 10^{-6}$[M]	2.3	3.6
[Ab] $\times 10^{+5}$ [M]	1.1	1.3

DISCUSSION

This study has been concerned with a test of the assumption that the extinction coefficient of a hapten bound to (its homologous) antibody is independent of the strength of the antibody-hapten bond formed. i.e. of the affinity of the antibody for the hapten. It was felt that any conclusions regarding this assumption would have to be based on a comparison of spectral binding measurements with those of an absolute method, and for this reason parallel experiments were performed using the equilibrium dialysis technique. In the antibody preparation used approximately 60% of the antibody sites present,(on the basis of the total number of sites determined by equilibrium dialysis) appeared to bind the hapten IN-3, 6S-2DNP with a constant extinction coefficient [Fig. 37].* This would tend to support the assumption that ϵ_b was a constant, however, in spite of this differences were noted in the amount of bound hapten, at a given total hapten concentration, calculated by spectrophotometry and by equilibrium dialysis. [Fig. 38] The difference in the results obtained by these two experimental methods is much more obvious in Fig. 39; however the effect is exaggerated by the ν_b vs $1/c$ plot. As can be seen from the results obtained for K_o and Ab, the fairly large difference in the two binding curves only leads to a 50 percent difference in average binding constants, and a 20% difference in the concentration of binding sites.

* The curvature of this plot above hapten concentrations of 6×10^{-6} M is of course due to the presence of free hapten in the solution.

It is difficult to assess the contribution of experimental errors to the differences shown in the binding curves of Figs. 38 and 39. The final values for b and c are based on calculations involving a maximum of five experimentally determined parameters i.e. three extinction coefficients, ϵ_{app}, ϵ_b and ϵ_f, and two correction factors for non-specific binding to the dialysis membrane and the NRG present. On this basis the deviation of the b vs b + c curves in Fig. 38 from a hypothetical mean would be about 5%, which would certainly fall well within experimental error for these experiments.

To explain any discrepancies that might arise in this or other systems between spectrophotometric results and those obtained by equilibrium dialysis, it is necessary to consider, at a molecular level, the nature of the interactions being observed. It would be expected that the effect on the spectrum of a dye hapten, due to its combination with (a homologous) antibody, would be the net result of many different and complex interactions which, because of the heterogeneous nature of the antibodies present, might well vary in their nature and extent throughout the antibody population. Thus, the extinction coefficient of the bound hapten might vary depending on the particular orientation of the hapten in the antibody site and on the nature of the forces (ionic, hydrophobic, van der Waals, hydrogen bonding, etc.) involved in each of the different types of possible associations.

In systems such as the one studied here, where the haptenic determinant group is attached to a relatively bulky substituent, the heterogeneity of the hapten-antibody reaction may not only be due to differences in the primary interaction between the haptenic determinant group and the antibody combining sites, but also to secondary interactions, which may occur because of conformational changes in the antibody molecule,

between the rest of the hapten molecule and groups on the antibody adjacent to its binding site. The extinction coefficient of the bound dye-hapten would be expected to be sensitive to these interactions, as well as to the different orientations of the haptenic moiety with respect to the rest of the dye molecule, which could be imposed by interactions with the antibody molecule. It is therefore conceivable that a heterogeneity of antibodies in regions about the actual combining site could lead to different spectral properties of the bound haptens. In the particular case studied, a variability in these interactions might affect, for example, the extent of ionization of the naptholic OH group of bound IN-3, 6S-2DNP molecules as well as the relative positions of the 2, 4-dinitrophenyl and napthol disulfonic acid residues.

It would, therefore, seem reasonable to consider the extinction coefficient of a bound hapten as an average value $\bar{\epsilon}_b$, defined by equation

$$\bar{\epsilon}_b = \frac{\sum_i \epsilon_{b,i} c_i}{\sum_i c_i}$$

where $\epsilon_{b,i}$ is the extinction coefficient of a hapten at a concentration c_i bound to an antibody molecule of a particular type (i). Practically, however, in spectrophotometric measurements, one is forced to assume a singularity of the extinction coefficient of the bound form of the hapten, while in reality, because of the heterogeneous nature of antibodies, the value of ϵ_b derived from measurements at relatively low hapten-antibody ratios will reflect primarily the contributions of antibodies with greatest affinity for the hapten. On the other hand, at higher hapten concentration, antibodies with lower binding constants and perhaps leading to different effects on the extinction coefficient of the bound form of the hapten, will also exert an effect and, therefore, the value used for ϵ_b may no

longer apply. If this interpretation were correct, it would follow that there might exist no direct relation between the overall strength of the antibody-hapten interaction and the magnitude of the spectral change induced in the dye-hapten on binding.*

Another indirect technique which has been used to evaluate antibody-hapten combination, is fluorescence quenching. Eisen et al (64) have reported good agreement between equilibrium dialysis and fluorescence quenching measurements using haptens containing the 2, 4-dinitrophenyl determinant and homologous antibodies. However the same authors also noted a variation in the efficiency of fluorescence quenching as a function of the amount of hapten bound in certain antibody preparations (85). Similarly, Saha et al (186), studying a different antibody hapten system than the above authors, found variations in binding data calculated by these two techniques and suggested that this might have been due to a lack of validity of the assumption that the extent of quenching was independent of antibody affinity.

In summary it would appear that all indirect methods available for the study of antibody hapten interactions suffer from a common drawback i.e. that they are sensitive to errors produced by the inherent heterogeneity of antibody preparations. However, it is also possible that in any given system the effect of antibody heterogeneity may be so small as to be negligible or not detectable by the method used. Therefore, each antibody-hapten system and technique should be considered separately and binding data compared to the results of an unequivocal technique, such as equilibrium dialysis, before final conclusions are drawn. The spectrophotometric technique has been profitably applied to kinetic studies of antibody-hapten

* A similar conclusion has been drawn by Klotz (156) from studies of the binding of dyes to bovine serum albumin.

systems (120, 121) and is particularly useful when strong absorption to dialysis membranes prevents the use of equilibrium dialysis techniques. The present study has shown that differences may exist in the extent of hapten binding calculated from spectrophotometric and equilibrium dialysis experiments. However it is felt that in view of the small magnitudes of these differences meaningful results can be obtained with this method in the 2, 4-dinitrophenyl system.

PART B

Kinetic and Equilibrium Studies of the Reaction between Antibodies Specific to the 2, 4-Dinitrophenyl Determinant Group and Two Dye-Haptens

INTRODUCTION

When the present investigation was initiated, only four studies of the kinetics of antibody-hapten reactions had been reported (119 - 121, 183). Of the various haptenic determinant groups that, along with their homologous antibodies, were considered to provide systems amenable to kinetic studies, the 2, 4-dinitrophenyl (DNP) group was thought to be the most suitable for further study for two reasons, i.e. (i) it had been shown that this determinant elicited a high titer antibody response in rabbits (184), and (ii) the reaction between such antibodies and homologous haptens had previously been studied by a combination of stopped flow and fluorescence quenching techniques (119).

It was pointed out in Part A of this Chapter that in certain hapten-antibody systems the occurrence of a spectral change in the hapten on binding can provide a useful method for following the extent of hapten-antibody combination. Unfortunately, however, the nature of the interactions that lead to these spectral changes are poorly understood. It was therefore decided to study, using the temperature-jump relaxation technique, the reaction between a single anti-DNP antibody preparation and two haptens, 1N-2S-4DNP and 1N-3,6S-2DNP, both possessing the same

(DNP) determinant group but differing in overall structure. It was hoped that the different orientations of the DNP group relative to other functional groups in these two haptens would have some effect on the hapten-antibody reaction which could then be related to the mechanisms of their combination and the spectral effects involved.

MATERIALS

Haptens

Three haptens IN-2S-4DNP, IN-2S-4pNP and IN-3, 6S-2DNP were used. Both IN-2S-4DNP and IN-3, 6S-2DNP contained the 2, 4-dinitrophenyl determinant group but differed in the relative positions of this residue with respect to the hydroxyl and sulfonic acid groups of the napthalene core. In addition IN-3, 6S-2DNP has a greater charge, due to its two sulfonic acid groups, than IN-2S-4DNP.

All of these dye haptens obeyed Beer's law up to concentrations of about 4×10^{-5} M, under the conditions used in this study.

The spectral properties of IN-2S-4DNP, IN-2S-4pNP and of IN-3, 6S-2DNP have been described in Chapters II and in Part A of this Chapter.

Borate buffers, $\mu = 0.1$, were used for all experiments at pH 8, and 9.5.

Proteins

A single anti-DNP rabbit globulin preparation was used in the equilibrium and kinetic experiments. This preparation was obtained by the procedure described in the first part of this chapter.

Flow Techniques and Relaxation Methods

As mentioned in the introduction to this thesis, two methods have been found to be especially suitable for measurements of the rates of reactions of antibodies with homologous haptens. In the stopped flow technique, solutions of the reactants are mixed by their rapid injection into a chamber which is constructed so as to allow efficient mixing of the reactants in a minimum length of time, of the order of a few milliseconds. The mixed reactants then exit into an observation tube where the rate at which the reaction proceeds can be measured by suitable techniques, such as spectrophotometry. One of the drawbacks of this method is that it is not possible to construct an apparatus that will allow observations of the mixed solutions in times less than about 3 milliseconds. This time lag, called the instrument dead time,* is due to the inherent slowness of the mixing process. Secondly, analytical observations are difficult to perform on flowing solutions due to complications introduced by deviations from mass-flow, cavitation etc. (187). The latter difficulties, can be overcome by the use of a "stopped flow" instrument which makes observations of the mixed reactants after their solution has ceased to flow, thus overcoming difficulties involved in making observations of a flowing solution. Moreover, as fluorescence quenching has been shown to be a very sensitive method for determining concentrations of bound and free hapten in the presence of antibody, when quenching of the antibody's fluorescence by a bound hapten occurs, (84),

* Dead times due to time lags in the electronic instrumentation of such an apparatus are usually much shorter than those due to mixing.

employing this technique with a "stopped flow" instrument allows very low concentrations, of the order of 10^{-7} M, of reactants to be used (119); at these low concentrations the half life time of an antibody-hapten reaction is significantly longer than the flow instrument's dead-time. This approach has been profitably used by Day et al (119) in kinetic studies of (DNP) hapten-antibody systems.

Relaxation methods differ fundamentally from flow techniques in that in the former the system under study is first allowed to reach equilibrium under controlled conditions of temperature, pressure, etc.. The equilibrium state is then very rapidly disturbed by an "instantaneous" perturbation i.e. within 10^{-6} - 10^{-8} sec, of one of the external parameters that affect it. If the perturbation occurs in a time which is much shorter than that required for re-equilibration of the system, observation of the rate at which the system adjusts itself to the new equilibrium allows determination of the rate constants for the elementary reaction steps involved in the re-equilibration process. Depending on whether the system to be studied undergoes, during reactions, a change in enthalpy, volume or partial molar polarizability a "sudden" perturbation of the temperature (188, 189) pressure (190), or of the electric field strength (191), can be used.

For the calculation of rate constants from relaxation measurements it is essential that the perturbation of the system produce only small deviations from the original equilibrium state. If this is true, then the rate equations describing the rate of attainment of the new equilibrium position can be linearized with respect to the time-dependent concentration differences involved (122). In other words the rate of disappearance of a small difference between the concentration of a

component and its equilibrium concentration is proportional to the magnitude of this difference.

For a single step reaction (of arbitrary order) the linearized rate can be expressed as

$$\frac{d\,Xi}{dt} = 1/\tau\ (\bar{X}i - Xi) \qquad [4]$$

where $Xi = Ci - Ci^\circ$ and $\bar{X}i = \bar{C}i - Ci^\circ$

Ci is defined as the concentration of the i'th component at any time t, $\bar{C}i$ is an equilibrium concentration which may be time-dependent and Ci° is a time-dependent reference concentration. The constant, τ, has the dimensions of time and is called the relaxation time. As stated above, relation (1) is only valid for the case $Ci \gg \bar{X}i - Xi$, i.e. for small deviations from equilibrium.

If a system such as

$$A + B \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C$$

is considered, then using the definitions given for Xi and $\bar{X}i$ one may write the concentrations of the components of the system in terms of the following relations, i.e.

$$C_A = C_A^\circ + X_A, \qquad C_B = C_B^\circ + X_B \qquad \text{etc.}$$

$$\bar{C}_A = C_A^\circ + \bar{X}_A, \qquad \bar{C}_B = C_B^\circ + \bar{X}_B \qquad \text{etc.}$$

and similarly $X_A = X_B = -X_C$, $\bar{X}_A = \bar{X}_B = \bar{X}_C$

Substitution of these values in the rate equation i.e.

$$\frac{-dC_A}{dt} = \frac{-dC_B}{dt} = \frac{dC_c}{dt} = k_{12}\,C_A C_B - k_{21} C_c \qquad [5]$$

results in an equation of the form of (1) above with

$$1/\tau = [k_{21} + k_{12}\,(\bar{C}_A + \bar{C}_B)] \qquad [6]$$

If equation [5] is rewritten in terms of the definitions for X_A in terms of C_A and C_A° etc. and in terms of the value for τ given in equation [6] then, if the value for $\bar{X}_A$ (or $\bar{X}_B$ or $\bar{X}_C$) is set equal to zero, integration of the resulting relation gives

$$X = Xo \quad \exp(-t/\tau) \qquad [7]$$

The relaxation time is thusethe time required for the concentration difference to be reduced to 1/e of its value at zero time. The values of the rate constants describing this reaction can then be calculated, as indicated in equation [6], from the dependence of the relaxation time on the equilibrium concentrations of the reactants.

If absorption spectrophotometry is used to follow the rate of attainment of equilibrium after perturbation, and ΔI is defined as the change in the absorbance of the system due to the perturbation, then ΔI is proportional to X. In terms of equation [7] therefore

$$I_t = Io \quad \exp(-t/\tau) \qquad [8]$$

If the reaction under consideration is a unimolecular conversion i.e. $A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} B$, then the relaxation time is given by the relation $1/\tau = k_{21} + k_{12}$ [9]

On the other hand for a bimolecular reaction in which one component is buffered i.e.

$$A + B \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C$$

where B is the "buffered" component, $1/\tau$ is given by

$$1/\tau = k_{21} + k_{12}(\bar{B}) \qquad [10]$$

It should also be noted that in the above examples, as is true for all systems, the expression for the relaxation time will include contributions from both the forward and reverse reaction steps. This is a reflection of the fact that no matter what the effect of the perturbation, in terms of whether it causes increased or decreased association, both forward and reverse reaction steps occur in the re-equilibration process.*

The Temperature-Jump Technique

As most chemical equilibria are temperature dependent, or can be coupled to temperature dependent reactions, the temperature jump technique is probably the most widely used of the various modern relaxation methods. The variation of the equilibrium constant of a reaction with respect to temperature is given by the van't Hoff relationship, i.e.

$$\frac{d \ln K}{dT} = + \frac{\Delta H}{RT^2} \qquad [11]$$

where K is the equilibrium constant for the reaction, ΔH is the enthalpy of reaction and R and T are the gas constant and absolute temperature, respectively. Any temperature change, therefore, imposed on an equilibrated reaction system possessing a finite ΔH, will force new equilibrium conditions and thus concentration changes upon the system.

The behavior of a given system (with a finite ΔH of reaction) after a temperature perturbation will depend on the relative rate of the temperature change itself and the rate of adjustment of the system to the new equilibrium at the higher temperature. Thus, if both these processes occur at similar rates no useful information can be obtained.

---

* These equations have all been derived from a mathematical analysis of relaxation phenomena by M. Eigen (122).

On the other hand, if the temperature rise occurs in a time which is much shorter than that required by the system to equilibrate at the new higher temperature, then any concentration differences produced will disappear in accordance with equation [e.g.4]. In other words if the temperature jump is sufficiently rapid it does not enter into the rate equations describing the re-equilibration reactions.

The Apparatus

A general outline of the temperature jump apparatus* is given in Figs. 40 and 41. A more detailed description may be obtained in reference (122).

The most important component of the instrument was a 0.1 μF capacitor (Plastic Capacitors Inc. Chicago) which was connected to the reaction cell through a (variable) spark gap and to a high voltage (5-30 Kv Neutronic) power supply. Discharge of this condenser produces the desired temperature perturbation (jump) in less than 10 microseconds. The cell, (Fig. 42) was constructed of sturdy plexiglass with a metal top and contained two rhodium plated electrodes separated by about 11 mm. The total (reaction) volume between the faces of the electrodes was about 1 cm, while about 15 ml of solution were necessary to fill the cell for an experiment. Two quartz rods, separated by about 1 cm, were cemented into the cell at right angles to the electrodes and midway between them. Observation of the reaction could thus be made spectrophotometrically by shining light of an appropriate wavelength through the quartz rods. The cell itself sits in a light-tight housing and is

* This apparatus was built in the workshop of the Max-Planck. Inst. for Physical Chemistry, Gottingen.

Figure 40

Schematic diagram of the temperature-jump apparatus.

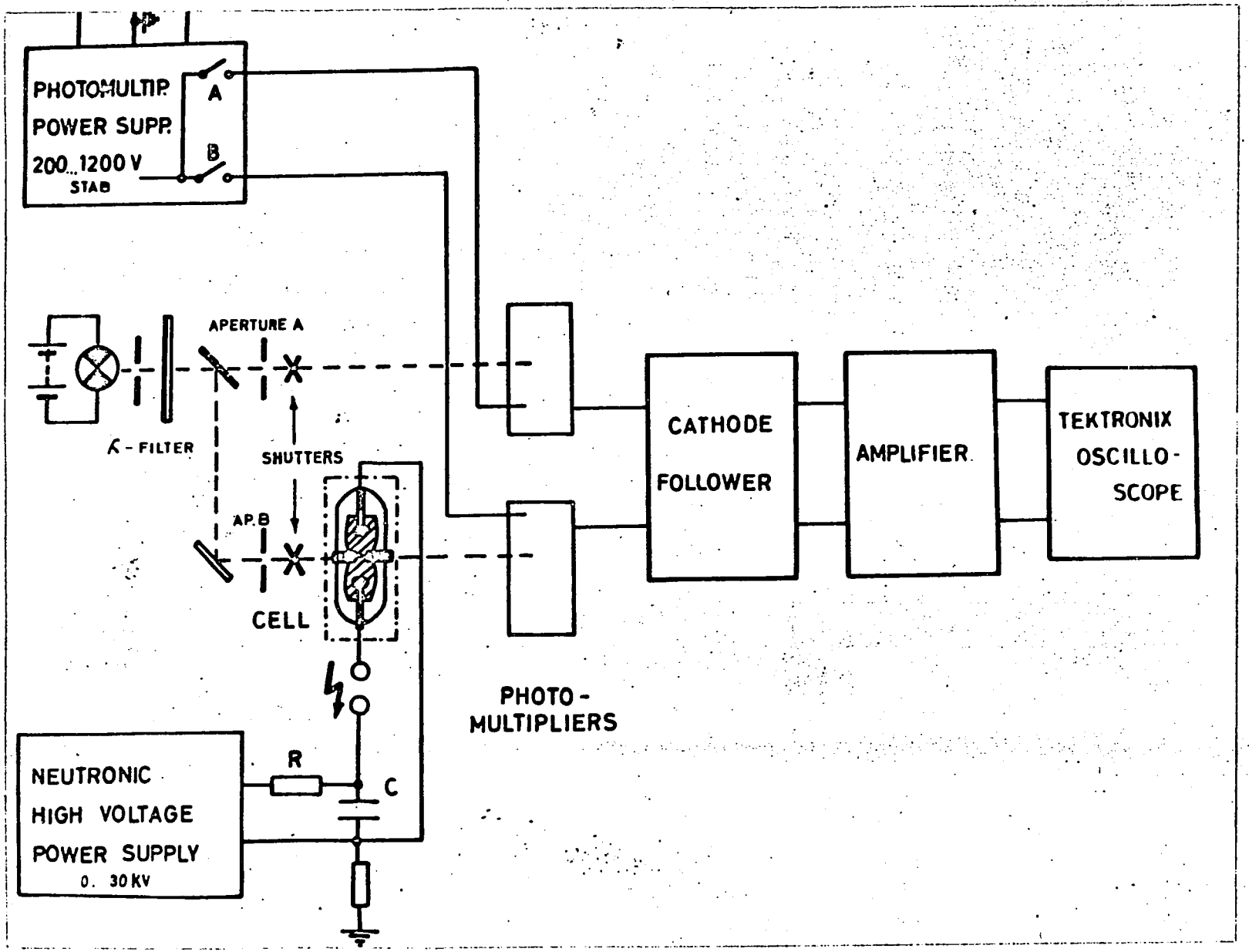
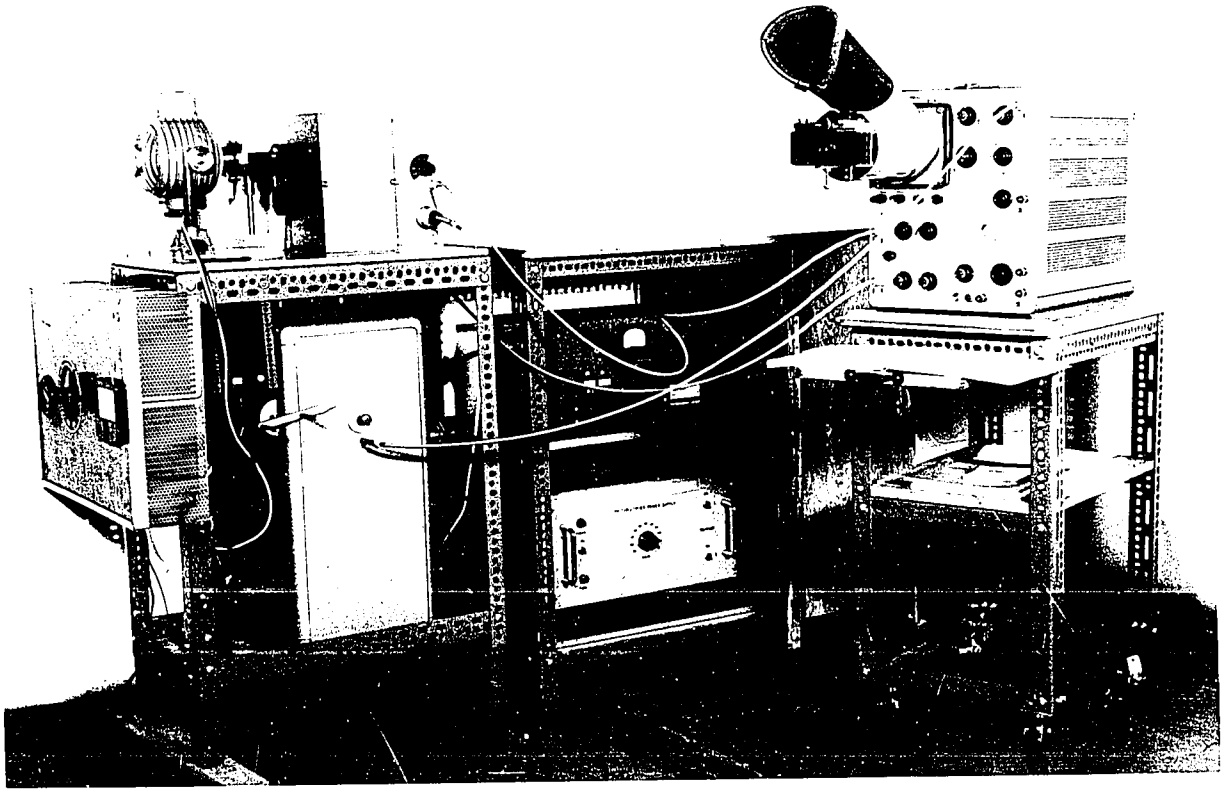

PHOTOMULTIP.
POWER SUPP.
200...1200 V
STAB
A
B
APERTURE A
K - FILTER
SHUTTERS
AP. B
CELL
PHOTO-
MULTIPLIERS
CATHODE
FOLLOWER
AMPLIFIER
TEKTRONIX
OSCILLO-
SCOPE
NEUTRONIC
HIGH VOLTAGE
POWER SUPPLY
0. 30 KV
R
C

Figure 41

Photograph of the temperature-jump apparatus



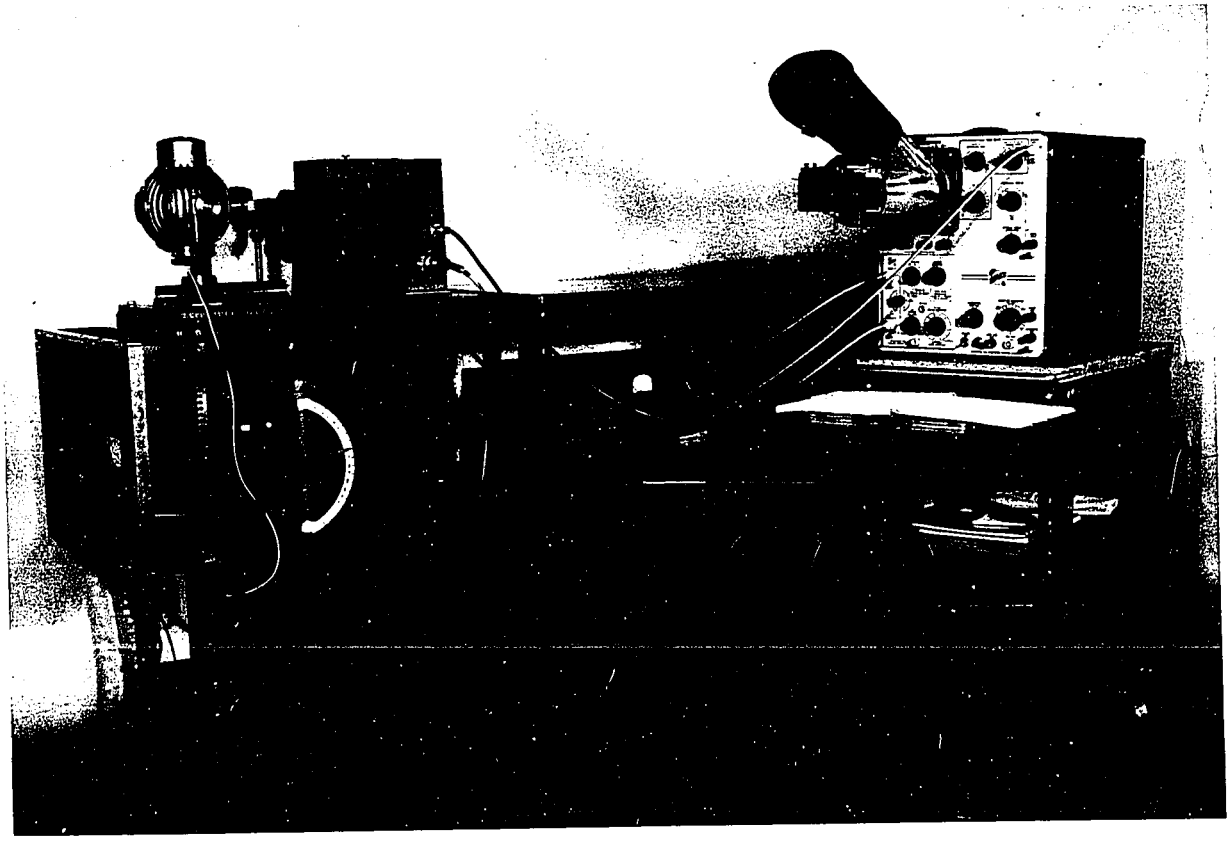
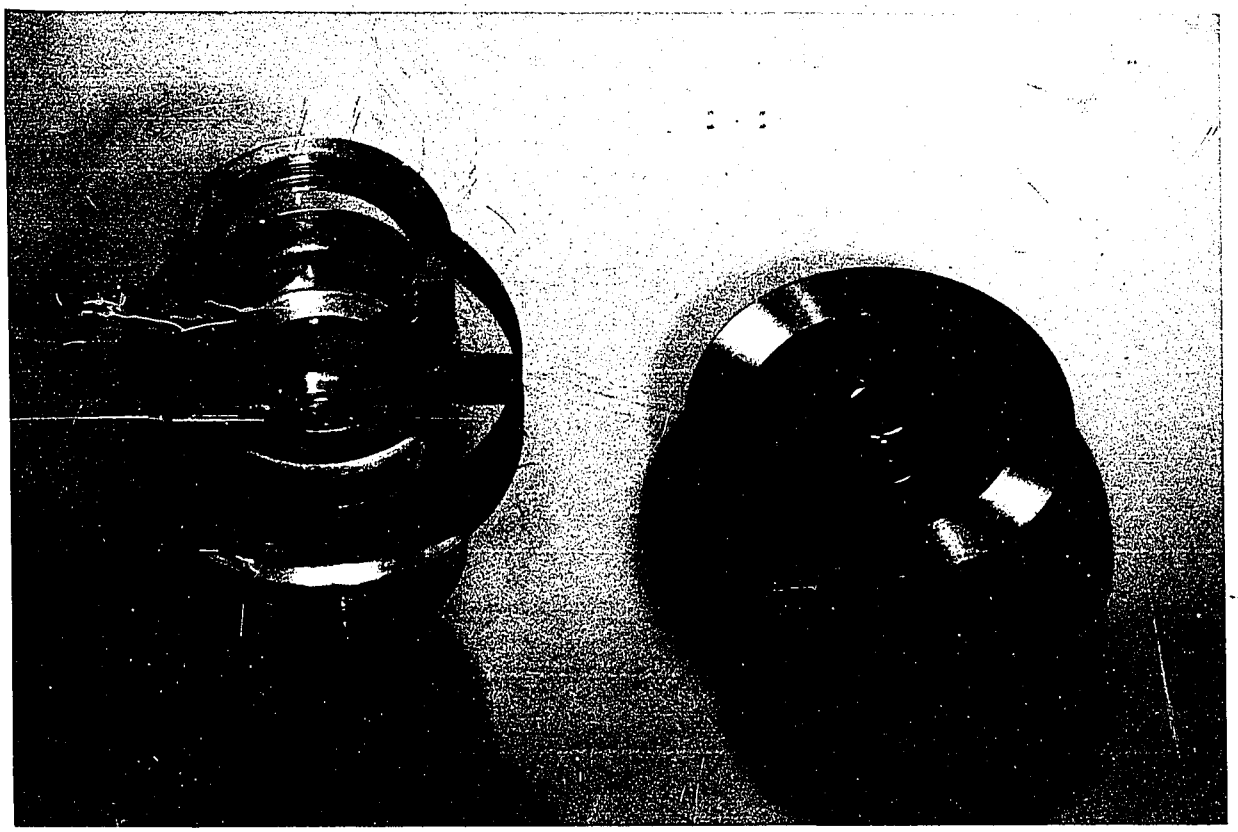
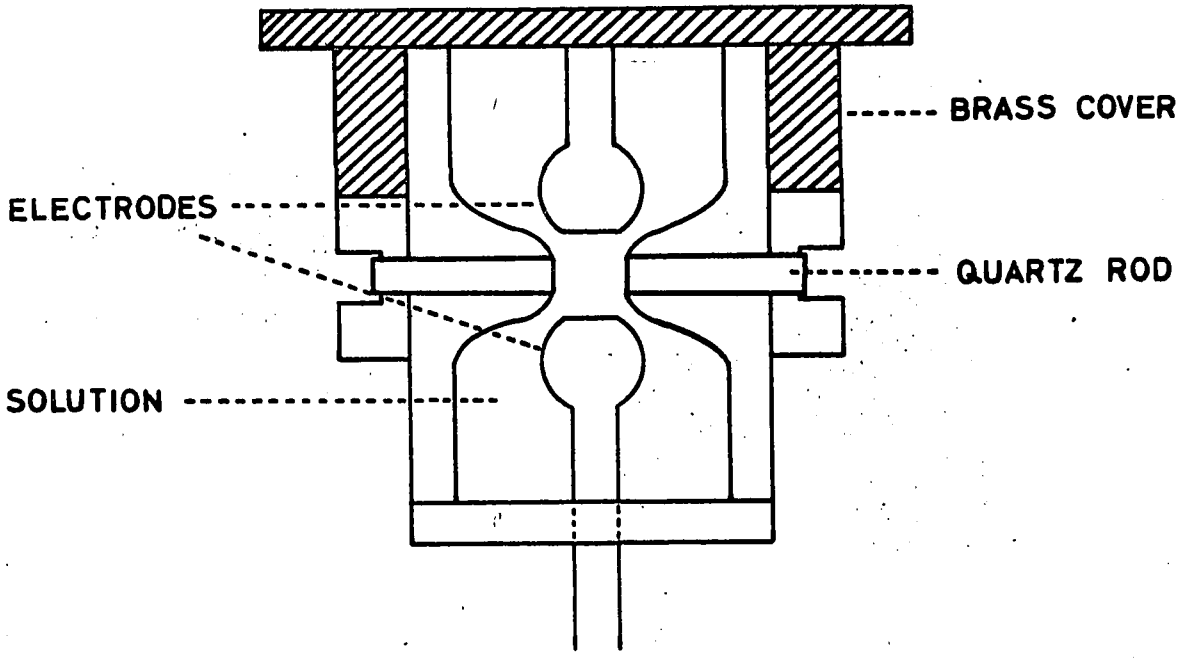
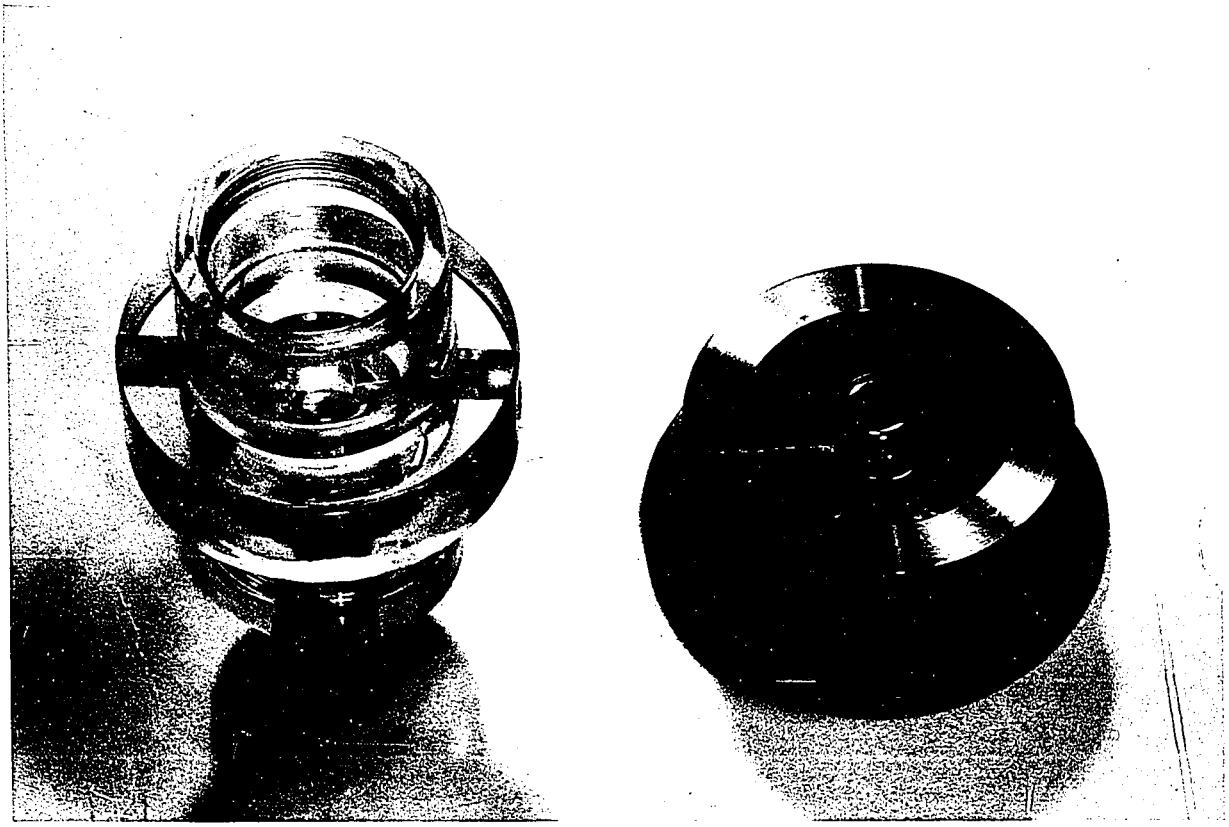


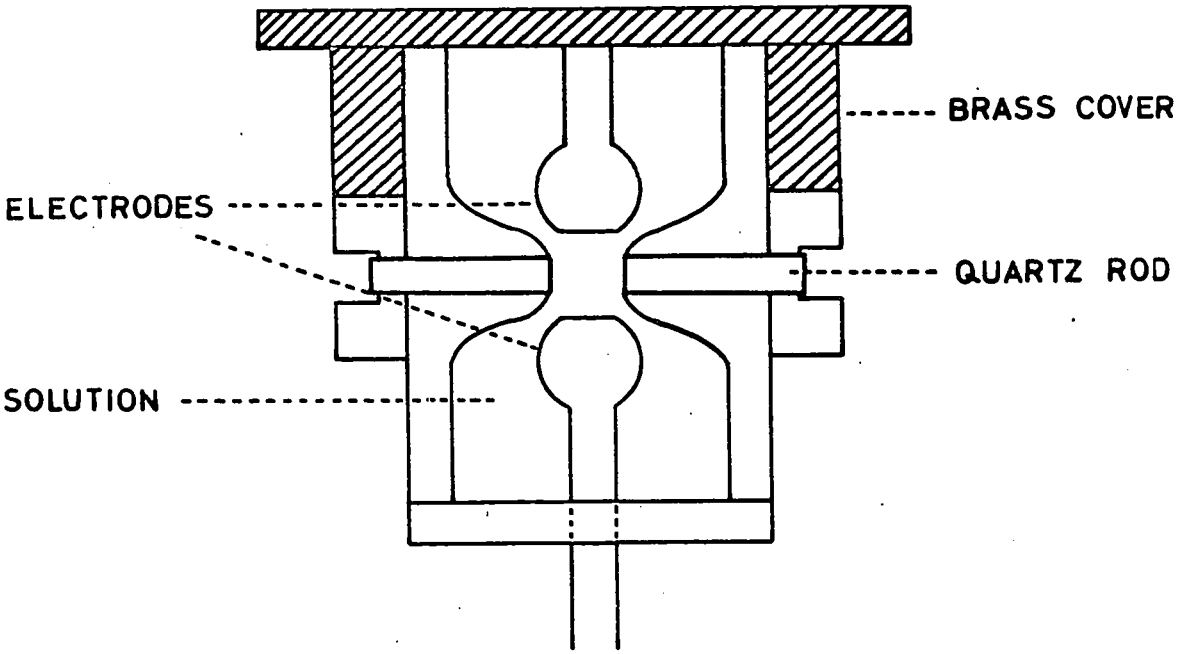
Figure 42

Photograph and schematic of the temperature-jump cell




BRASS COVER
ELECTRODES
QUARTZ ROD
SOLUTION




BRASS COVER
ELECTRODES
QUARTZ ROD
SOLUTION

maintained at a constant temperature by an insulated jacket which is connected to a constant temperature bath.

The light source was a 6.6A/T2 1/2Q/C1 45 watt Sylvania quartz iodine airport marker lamp, powered by 12 volt auto batteries, which was used in conjunction with an interference filter. After passing through the filter the light beam was split by a half silvered mirror, one part going through air and producing a reference signal, and the second beam passing through the cell. The two beams impinged on two photomultipliers (RCA 1P28) powered by a stabilized power supply. The signal from the photomultipliers was fed into a Tektronix Type D high gain differential preamplifier connected to a Tektronix 531A oscilloscope.

The sequence of operations during an experiment was as follows: The cell containing the solution under study was placed in the thermostated cell holder and allowed to reach the temperature of the thermostat. The dark currents of the two photomultipliers were balanced against each other by means of a slide wire resistance connected to the two cathode followers. The light source was then turned on and the differential signal from the photomultipliers minimized by adjustment of the iris diaphragms controlling the size of apertures A and B. At this point the high voltage power supply could be brought into the circuit and the condenser charged to the desired voltage through the 400 mega-ohm resistor "R". The spark gap was set so that the condenser would discharge at a voltage of about 30 Kv, simultaneously raising the temperature of the solution between the electrodes and triggering the oscilloscope.

Flow Measurements

A Durrum-Gibson stopped flow apparatus (Durrum Corp., Palo Alto, California) became available towards the end of this study and was used to perform exploratory experiments with the hapten IN-3, 6S-2DNP and anti-DNP antibodies.

Briefly, this apparatus consists of two 5 ml syringes which are filled with the two reactants and which are connected to a mixing chamber. This chamber in turn empties directly into a cuvette which is connected to the "stop-syringe". The two syringes containing the reactants are driven by a hydraulic ram by which a rapid impulse is applied to both syringe plungers simultaneously; each impulse results in the displacement of about 0.2 ml of solution from each syringe through the mixing chamber and into the cuvette. Visible or ultraviolet light from a monochromator (Baush and Lomb) passes through the cuvette and the rate of reaction is followed by monitoring the absorbance (or fluorescence) of the solution with a photomultiplier tube whose signal is fed into a Tektronix storage oscilloscope. The time sweep of the oscilloscope is begun by forcing fresh reactants into the cuvette. This in turn expels the "old" solution into the stop syringe and trips a switch that triggers the sweep. The dead time of the instrument (about 3 milliseconds) thus represents the time necessary to refil the cuvette with a fresh solution of completely mixed reactants. The mixing chamber, cuvette and reactant syringes are maintained at a constant temperature by circulating water from a constant temperature bath through the housing of the instrument.

Binding Measurements

Equilibrium binding measurements were performed at 25°C using the spectrophotometric technique described in Part A of this Chapter.

RESULTS

A) Equilibrium Measurements

The effects of rabbit anti-DNP antibodies (at a concentration of 3.5×10^{-5} M) on (i) a 3.1×10^{-6} M solution of IN-2S-4DNP at pH 8 and pH 4.6, (ii) on a solution of IN-2S-4pNP ($\sim 3 \times 10^{-6}$ M) at pH 8, (iii) and on a 6.8×10^{-6} M solution of IN-3, 6S-2DNP also at pH 8, (antibody 1.7×10^{-6} M) are shown in Figures 43, 44 and 35 respectively. It will be noted from these figures that the results at pH 8 for these three haptens are quite similar, i.e. binding to antibody causes a shift of the spectrum of the dye to lower wavelengths, to one similar to that of the free hapten in its protonated form.

The results of the addition of varying concentrations of antibody to a 3.1×10^{-6} M solution of IN-2S-4DNP at pH 9.5 is shown in Fig. 45. On the other hand, addition of an NRG preparation did not lead to any effect on the spectrum of these dyes under these conditions. It will be noted that two isosbestic points can be observed in Fig. 45. This is indicative of the presence of three forms of the hapten in these solutions (vide infra) and was also taken to indicate that the spectrophotometric method for determining binding data should provide meaningful results in this system. Unfortunately, the extensive binding of IN-2S-4DNP to a dialysis membrane, even at this high pH, precluded verification of this assumption.

Figure 43

Effect of anti-DNP antibodies (3.5×10^{-5} M) on the spectrum of a 3.1×10^{-6} M solution of 1N-2S-4DNP. Curve #1 - Buffer pH 4.6 $\mu = 0.1$. Curve #2 - Buffer pH 4.6 plus antibody, Curve #3 - Buffer pH 8, $\mu = 0.1$, Curve #4 - Buffer pH 8 plus antibody.

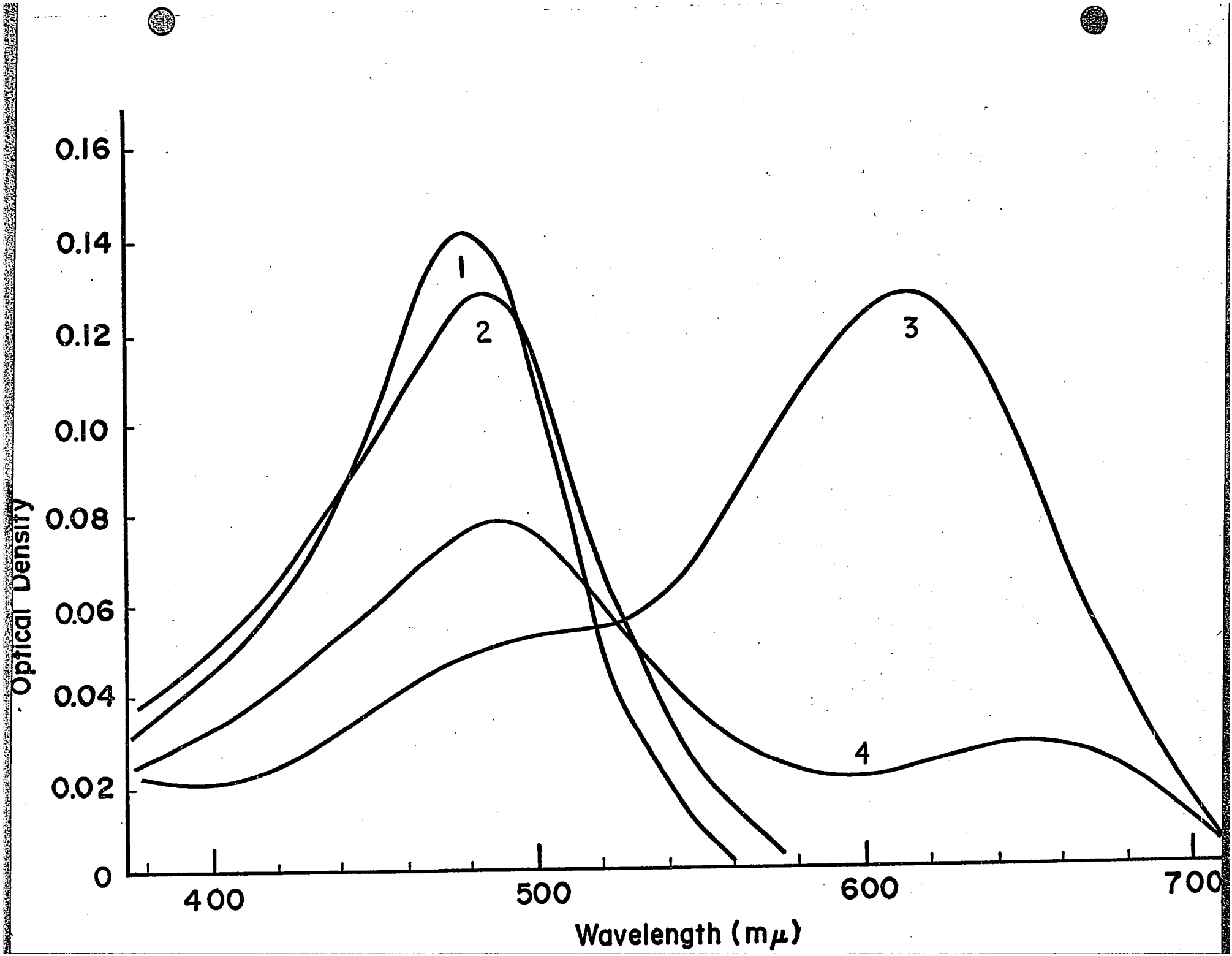

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Optical Density
1
2
3
4
400
500
600
700
Wavelength (mμ)

Figure 44

Effect of anti-DNP antibodies on the spectrum of IN-2S-4pNP at pH 8 μ=0.1. Curve #1 - Buffer only, Curve #2 - Buffer plus 5×10^{-6} M antibody.

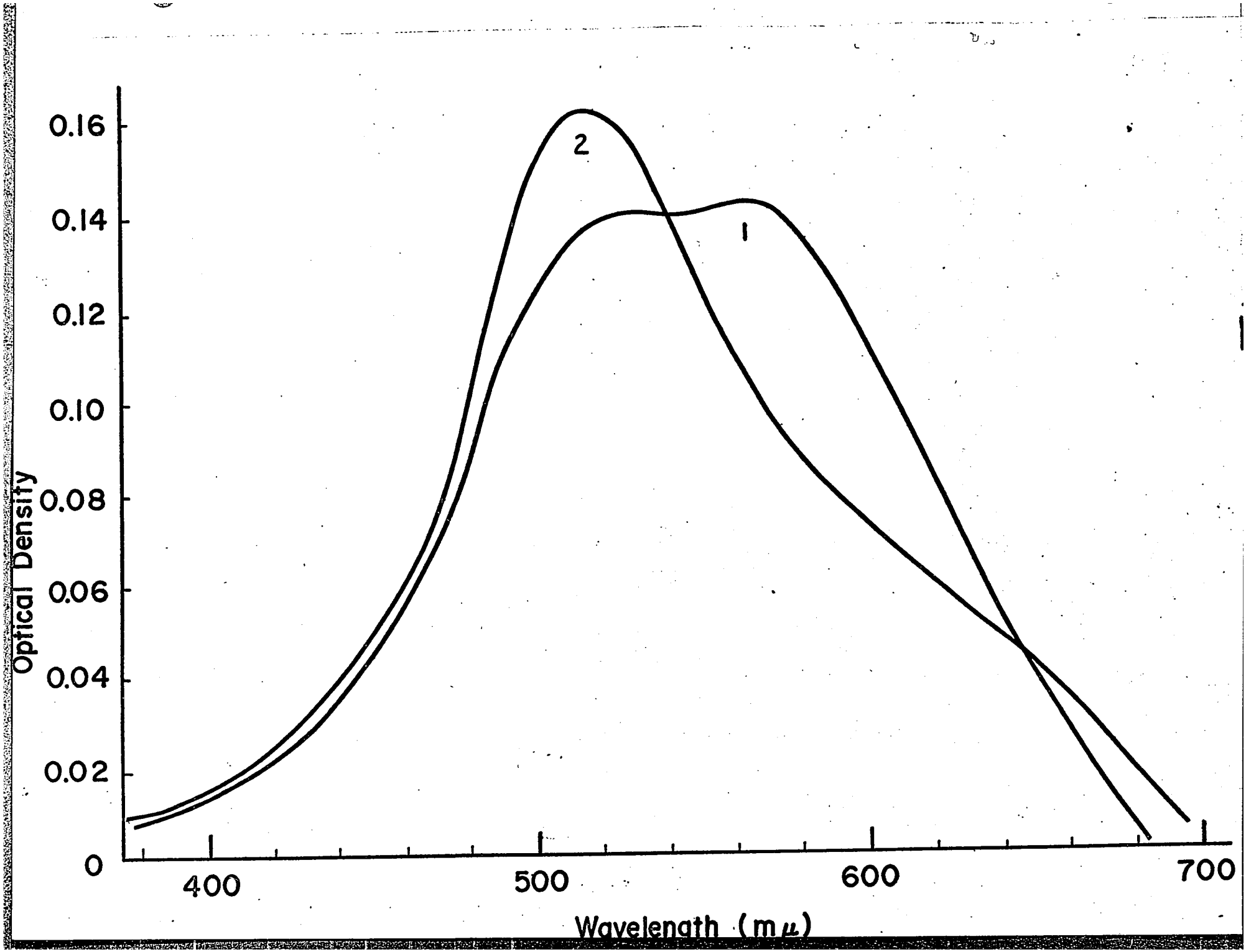
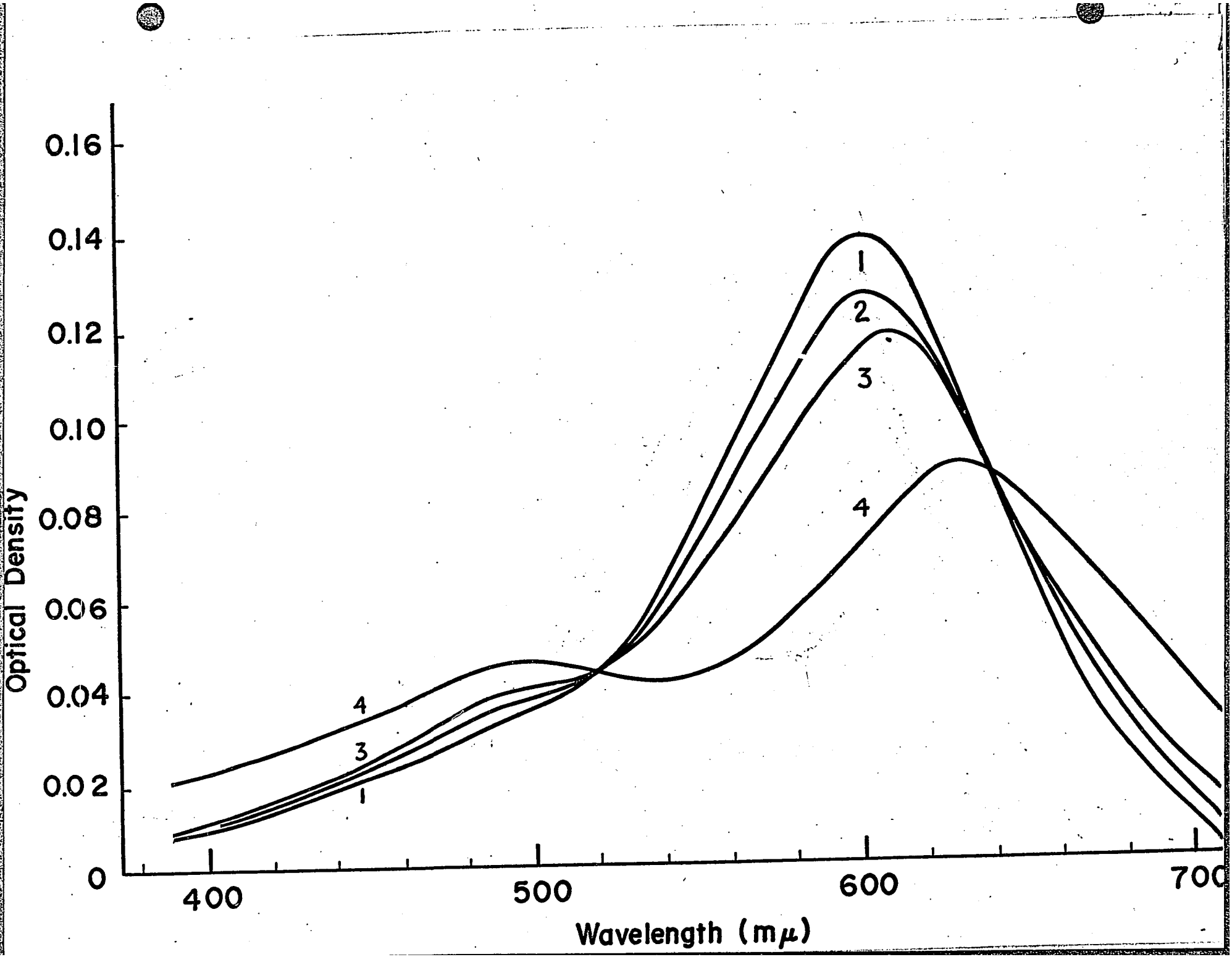

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Optical Density
2
1
400
500
600
700
Wavelength (mμ)

Figure 45

Effect of varying concentrations of anti-DNP antibodies on the spectrum of a 3.1×10^{-6} M solution of IN-2S-4DNP pH 9.5 $\mu = 0.1$. Curve #1 - Buffer only, Curve #2 - 1×10^{-7} M antibody, Curve #3 - 2×10^{-7} M antibody, Curve #4 - 1×10^{-6} M antibody


0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Optical Density
400
500
600
700
Wavelength (mμ)
1
2
3
4
4
3
1

The result of adding this antibody preparation (4×10^{-6} M) to a solution of IN-2S-4DNP (4.6×10^{-6} M) at pH 5 μ = 0.02 is shown in Fig. 46. In the same figure is also shown the effect of BSA (3.6×10^{-6} M) on the dye under the same conditions. A comparison of the different effects of antibody and BSA on IN-2S-4DNP at pH and 9.5 (for the effect of BSA on the dye at pH 9.5 please see Ch. II, Fig. 8) indicates that the interactions of these proteins with this dye are completely different.

The large spectral shift of IN-2S-4DNP and IN-3, 6S-2DNP in the presence of antibodies was used, as described in Part A of this Chapter, to obtain a measure of the concentrations of bound and free forms of the hapten in antibody-hapten solutions. Values for the extinction coefficients of the bound forms of IN-3, 6S-2DNP at pH 8 and of IN-2S-4DNP at pH 9.5 were obtained in a similar manner to that described previously (Figs. 47, 48). The values so obtained for ϵ_b, as well as the corresponding values for ϵ_f, are given in Table VII.

Table VII

	ϵ_b / $\times 10^{-4}$ M^{-1} cm^{-1}	ϵ_f / $\times 10^{-4}$ M^{-1} cm^{-1}
IN-3, 6S-2DNP		
pH 8, 486 mμ	2.84	4.11
IN-2S-4DNP		
pH 9.5, 615 mμ	2.32	4.24

Binding experiments were performed at pH 8 with IN-3, 6S-2DNP, and at pH 9.5 with IN-2S-4DNP, using anti-DNP γ-globulin solutions which were 0.63×10^{-5} M and 1.69×10^{-5} M in total protein, respectively. The results of these measurements are given in Tables VIII (IN-3, 6S-2DNP)

Figure 46

Effect of anti-DNP antibody and of BSA on the spectrum of IN-2S-4DNP at pH 5 $\mu = 0.02$. Curve #1 - Buffer pH 5, Curve #2 - Buffer plus anti-DNP (4×10^{-6} M), Curve #3 - Buffer plus BSA (3.6×10^{-6} M)

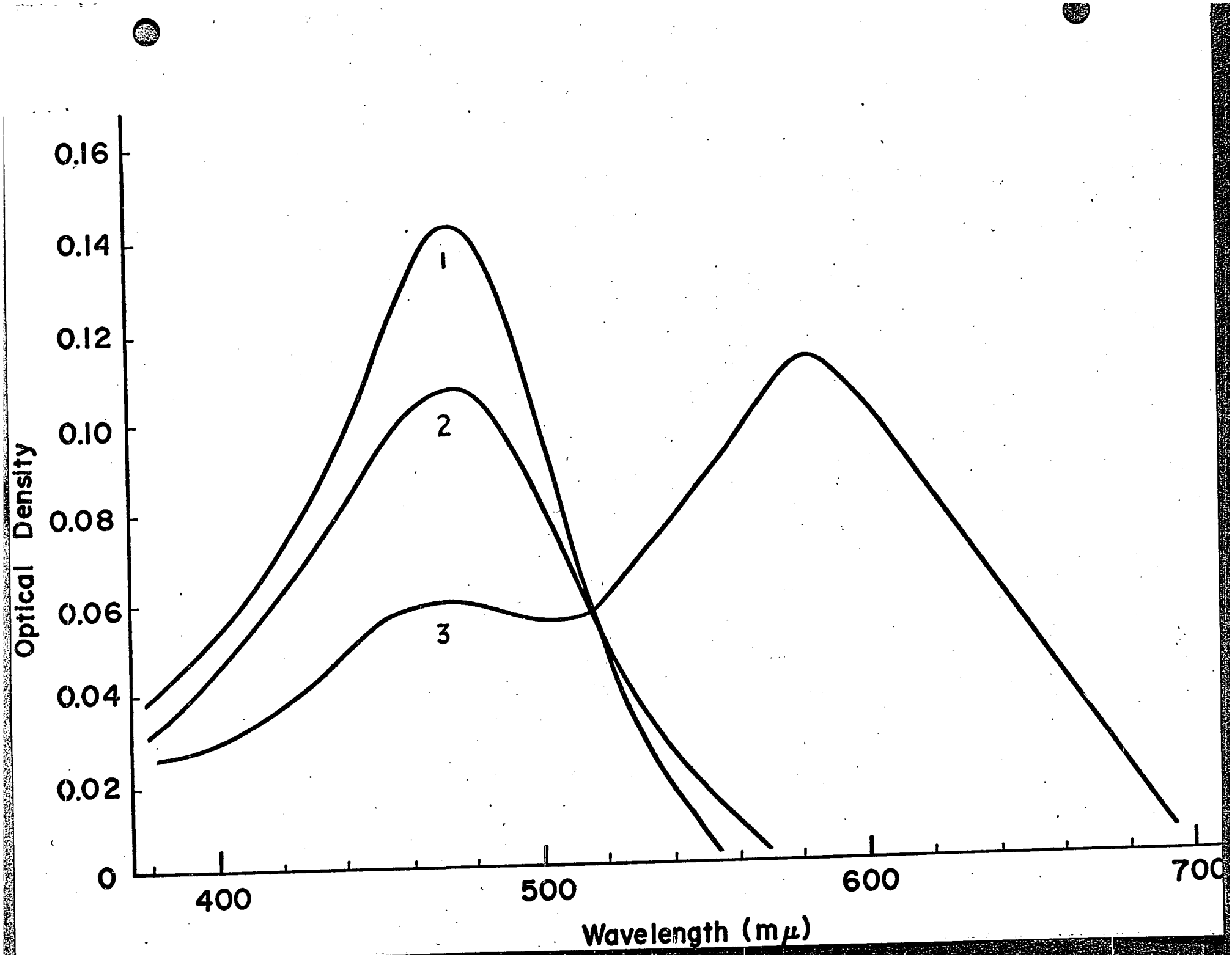

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Optical Density
1
2
3
400
500
600
700
Wavelength (mμ)

Figure 47

Evaluation of ϵ_b for IN-3, 6S-2DNP bound to anti-DNP antibody at pH 8 μ = 0.1.

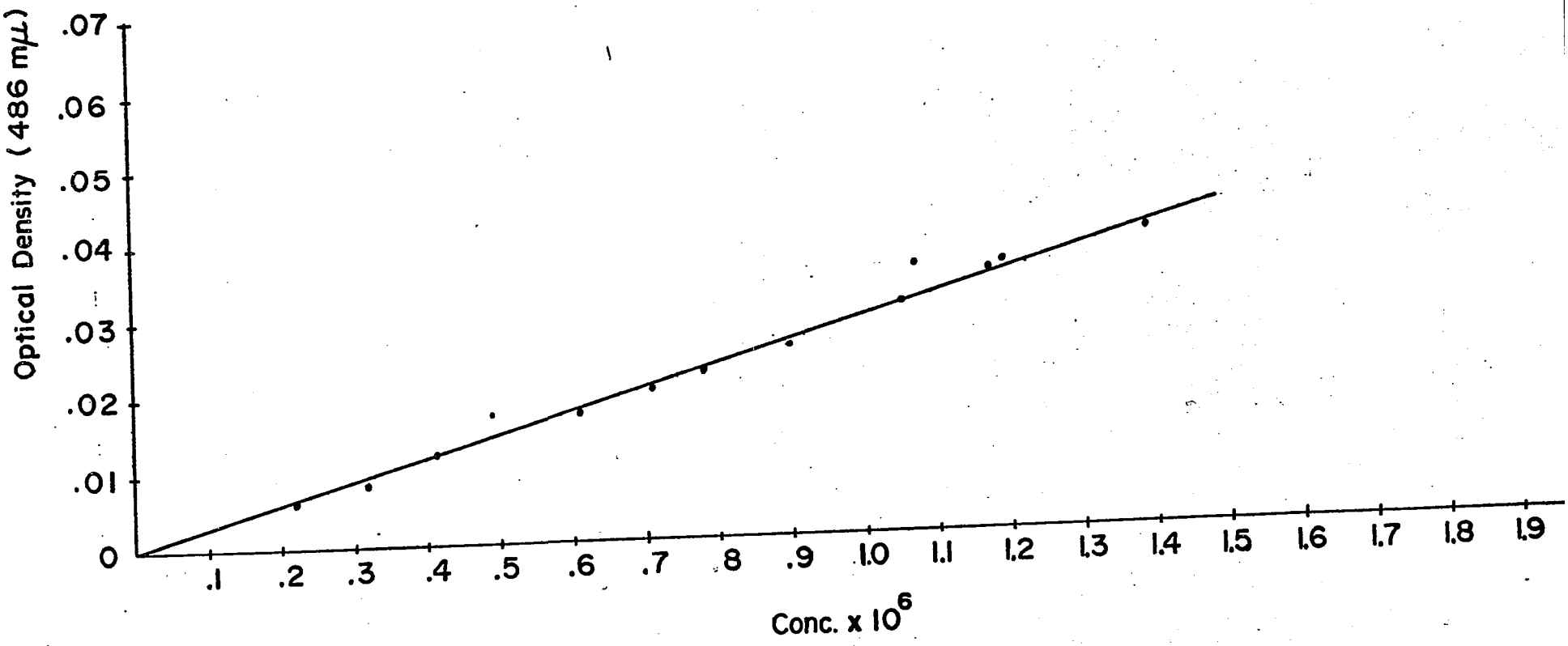
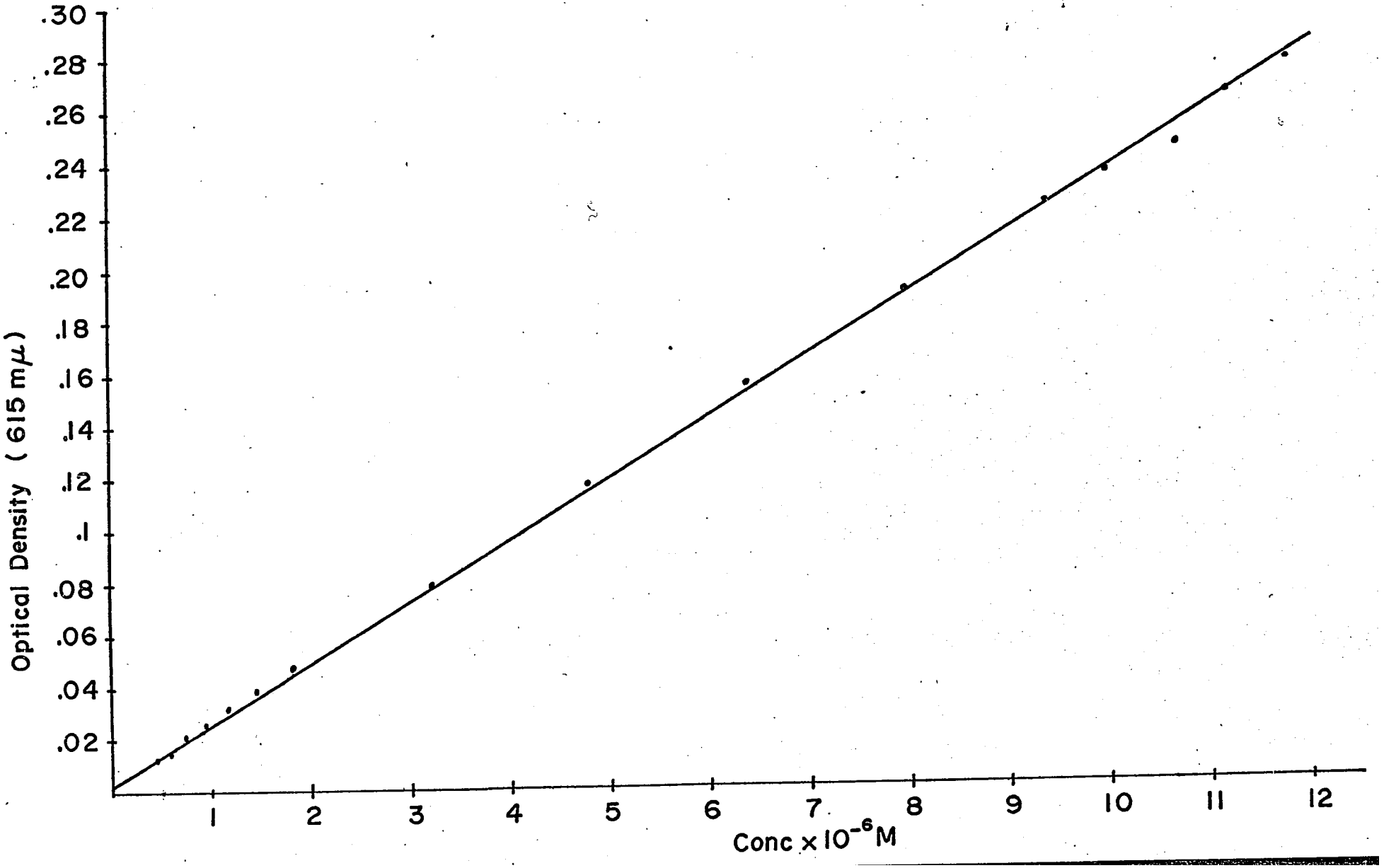

Optical Density (486 mμ)
.07
.06
.05
.04
.03
.02
.01
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
Conc. x 10^6

Figure 48

Evaluation of ϵ_b for IN-2S-4DNP bound to anti-DNP antibody at pH 9.5, μ= 0.1.


.30
.28
.26
.24
.22
.20
.18
.16
.14
.12
.1
.08
.06
.04
.02
Optical Density (615 mμ)
1
2
3
4
5
6
7
8
9
10
11
12
Conc x 10⁻⁶M

and IX (IN-2S-4DNP) and are represented in terms of a $1/b$ vs $1/c$ plots (Eg. [3], Ch. III, Part A) in Figs. 49 (IN-3, 6S-2DNP) and 50 (IN-2S-4DNP). The values of b and c calculated for the IN-3, 6S-2DNP experiments were adjusted for non-specific binding by normal γ-globulins present in the antibody preparation. This was not done in the case of IN-2S-4DNP because of the previously mentioned difficulty in performing equilibrium dialysis experiments with this hapten.

The values calculated for K_o and [Ab] from Figs. 49 and 50 are listed in Table X.

Table X

	Total Glob.	K_o	[Ab]
	Conc x 10^5 M	x 10^{-6} M^{-1}	x 10^6 M
IN-3, 6S-2DNP	0.63	3.2	3.6
IN-2S-4DNP	1.69	10	9.1

Adjustment of the value for [Ab] found from experiments with the IN-3, 6S-2DNP system using solutions 0.63 x 10^{-5} M in protein to a total protein concentration of 1.69 x 10^{-5} M, identical to that used for IN-2S-4DNP measurements, leads to a value of 9.7 x 10^{-6} M. The difference between this value and that determined from experiments with IN-2S-4DNP is considered a reflection of the uncertainties involved in the extrapolation of the $1/b$ vs $1/c$ plot to the $1/b$ axis.

Kinetic Studies

A) Determination of the Temperature Rise in the Temperature Jump Apparatus

The magnitude of the temperature jump was determined by measurement of the voltage change, as seen on the oscilloscope, produced

Table VIII

Binding of IN-3,6S-2DNP by Anti-DNP Antibodies

pH 8

IN-3,6S-2DNP added $\times 10^6[M]$	ϵ_{app}(486mμ) $\times 10^{-4}[M^{-1}cm^{-1}]$	β	b $\times 10^6[M]$	c $\times 10^6[M]$	$1/_b$ $\times 10^{-6}[M^{-1}]$	$1/_c$ $\times 10^{-6}[M^{-1}]$
1.39	2.81	0.977	1.36	0.03	0.735	33.0
1.68	2.74	0.922	1.55	0.13	0.645	7.69
1.95	2.72	0.907	1.77	0.18	0.565	5.55
2.02	2.72	0.907	1.83	0.19	0.546	5.26
2.14	2.61	0.822	1.76	0.38	0.568	2.63
2.70	2.52	0.752	2.03	0.67	0.493	1.49
2.88	2.43	0.682	1.96	0.92	0.510	1.09
3.16	2.45	0.698	2.21	0.95	0.452	1.05
3.31	2.39	0.681	2.15	1.16	0.465	0.86
4.23	2.25	0.543	2.30	1.93	0.435	0.518
5.35	2.13	0.450	2.41	2.94	0.415	0.340
6.20	2.08	0.410	2.54	3.66	0.393	0.273
7.06	2.04	0.380	2.68	4.38	0.373	0.228
8.35	1.93	0.295	2.46	5.89	0.407	0.170
9.10	1.92	0.287	2.61	6.49	0.383	0.150
10.27	1.88	0.256	2.63	7.64	0.380	0.131

Table IX

Binding of IN-2S-4DNP by Anti-DNP Antibodies

pH 9.5

IN-2S-4DNP added $\times 10^6$ [M]	ϵ_{app} (615 mμ) $\times 10^{-4}$[$M^{-1}cm^{-1}$]	β	b $\times 10^6$[M]	c $\times 10^6$[M]	$1/_b$ $\times 10^{-6}$[M^{-1}]	$1/_c$ $\times 10^{-6}$[M^{-1}]
4.79	2.36	0.98	4.69	0.10	0.215	10.0
5.18	2.38	0.97	5.01	0.17	0.200	6.0
5.80	2.40	0.96	5.55	0.25	0.180	4.0
6.39	2.47	0.92	5.88	0.51	0.170	1.95
7.16	2.55	0.88	6.25	0.91	0.160	1.10
7.96	2.69	0.81	6.45	1.51	0.155	0.663
9.39	2.80	0.70	6.56	2.83	0.153	0.354
10.7	2.88	0.64	6.85	3.75	0.146	0.267
11.2	3.06	0.62	6.91	4.29	0.144	0.234
11.8	3.13	0.58	6.85	4.95	0.146	0.202
12.7	3.23	0.53	6.73	5.97	0.149	0.176
14.3	3.29	0.50	7.15	7.15	0.140	0.140
16.8	3.41	0.43	7.22	9.58	0.139	0.104

Figure 49

Binding data for the IN-3, 6S-2DNP - anti-DNP system at pH 8, μ = 0.1.

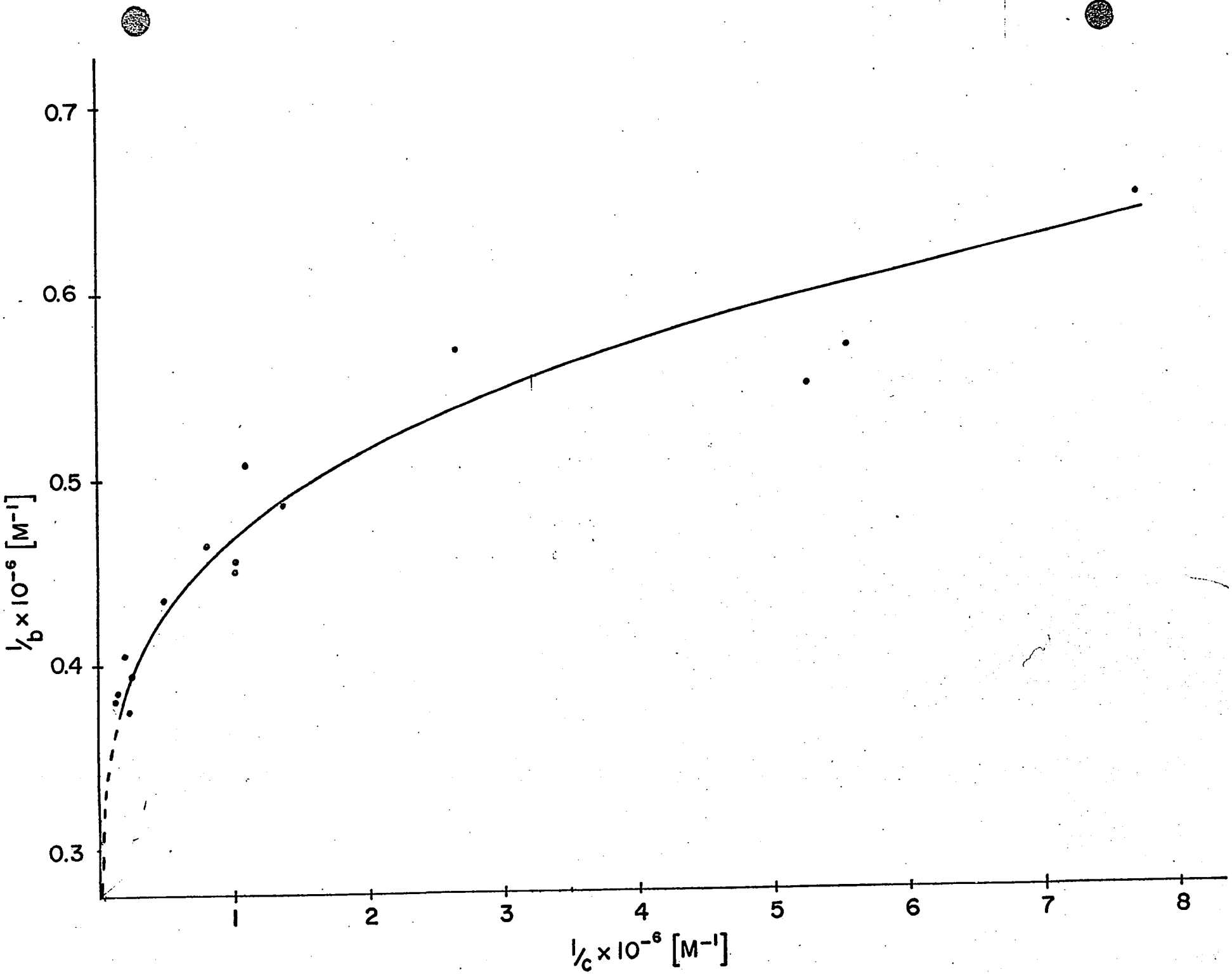
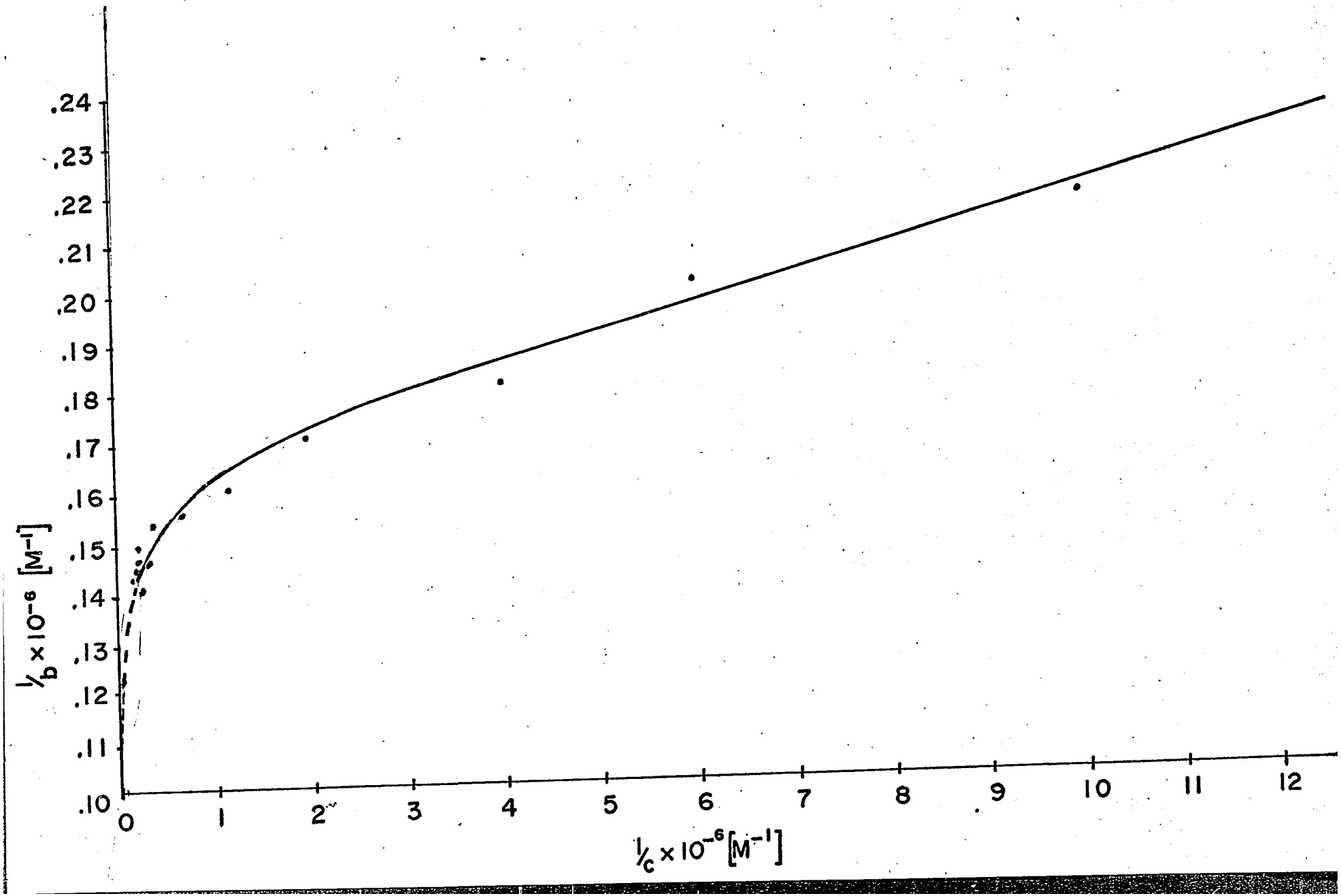

0.7
0.6
0.5
0.4
0.3
1/b x 10^-6 [M^-1]
1
2
3
4
5
6
7
8
1/c x 10^-6 [M^-1]

Figure 59

Binding data for the IN-2S-4DNP - anti-DNP system at pH 9.5, μ = 0.1 .


.24
.23
.22
.21
.20
.19
.18
.17
.16
.15
.14
.13
.12
.11
.10
1/b x 10^-6 [M^-1]
0
1
2
3
4
5
6
7
8
9
10
11
12
1/c x 10^-6 [M^-1]

by discharging the condenser through a buffered solution (pH 7.5) of the azo dye 1-Napthol 4-(p-azoarsanilic acid), 2-sulfonic acid. The optical density of this same solution at various temperatures was then determined in a Beckman DU spectrophotometer fitted with a thermostated cell holder. Similarly, a relation between voltage on the oscilloscope and optical density of a solution in the temperature jump cell was obtained by filling the cell with solutions of known optical densities. These measurements showed that under the conditions used in this study, i.e. ionic strength of 0.1 and a fixed spark gap setting of about 9 mm, discharge of the condenser caused a temperature jump of 6°C in 6 to 10 microseconds.

All binding studies were performed at 25°C; for this reason the temperature of the thermostat was set at 19°C, so that the final temperature in the reaction cell after the temperature jump would be 25°C. In this way the results of the equilibrium studies could be used to obtain values of the equilibrium concentrations of antibody and hapten present after the re-equilibration process.

B) Reaction of IN-3, 6S-2DNP with Anti-DNP Antibody

Temperature jump experiments were done with this hapten in Borate buffer, pH 8, $\mu = 0.1$, at which the free dye is mainly in the deprotonated form. No relaxation effects were observed in solutions of this hapten in buffer alone or in normal γ-globulin solutions of the same total protein concentrations as those containing anti-DNP antibody.

The concentrations of antibody and hapten in the solutions used for these experiments and the average relaxations times calculated from experiments with these solutions are given in Table XI; Example of a relaxation effect in this system is shown in Fig. 51. Relaxation effects

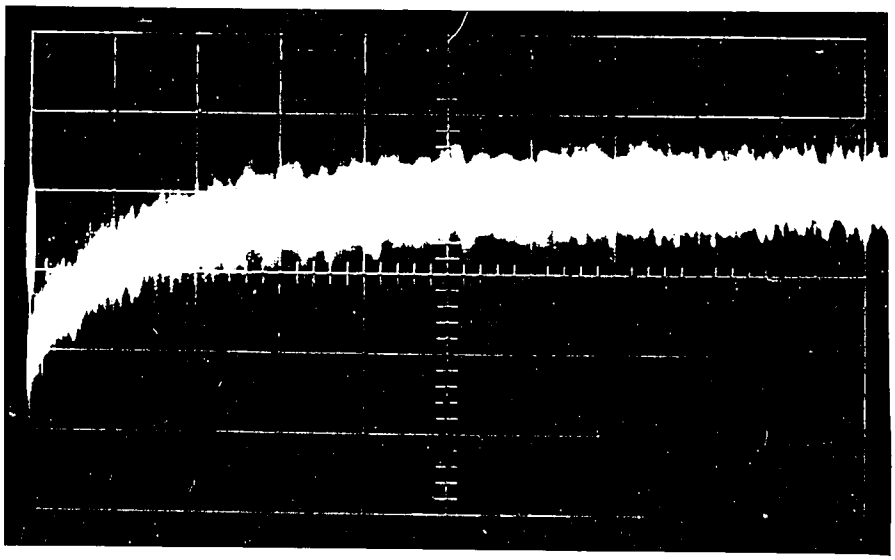
Table XI

Relaxation Experiments with the IN-3, 6S-2DNP——Anti-DNP System at pH 8

[Ab] total $\times 10^6$ [M]	[H] total $\times 10^6$ [M]	[H] bound $\times 10^6$ [M]	$[\bar{Ab}]$ $\times 10^6$ [M]	$[\bar{H}]$ $\times 10^6$ [M]	$[\bar{Ab}+\bar{H}]$ $\times 10^6$[M]	τ [sec]	$1/\tau$ [sec^{-1}]
3.40	2.77	1.75	1.65	1.02	2.67	0.285	3.51
3.40	4.86	2.48	0.92	2.38	3.30	0.185	5.41
3.54	8.59	2.77	0.77	5.82	6.59	0.180	5.55
3.40	8.60	2.71	0.69	5.89	6.58	0.195	5.13
3.40	10.9	2.82	0.58	8.08	8.66	0.150	6.66
1.75	15.4	1.47	0.28	13.9	14.2	0.152	6.58
3.16	18.9	2.78	0.38	16.1	16.5	0.119	8.40

Figure 50

Example of a relaxation curve in the IN-3, 6S-2DNP - anti-DNP system at pH 8, μ = 0.1. Sweep - 0.1 sec/cm.



were monitored at 590 mμ ; observations at 485 mμ gave identical values for relaxation times, although as was expected, the curvature of the relaxation curve was reversed with respect to the time axis.

The values obtained for $1/\tau$ are plotted as function of the sum of the concentrations of free antibody sites and free hapten present in Fig. 52. As can be seen the data fall on a straight line, indicating that the reaction can be represented by an expression of the form

$$Ab + Hp \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AbHp$$

The corresponding values for k_{12} and k_{21}, calculated on the basis of equation [6] are

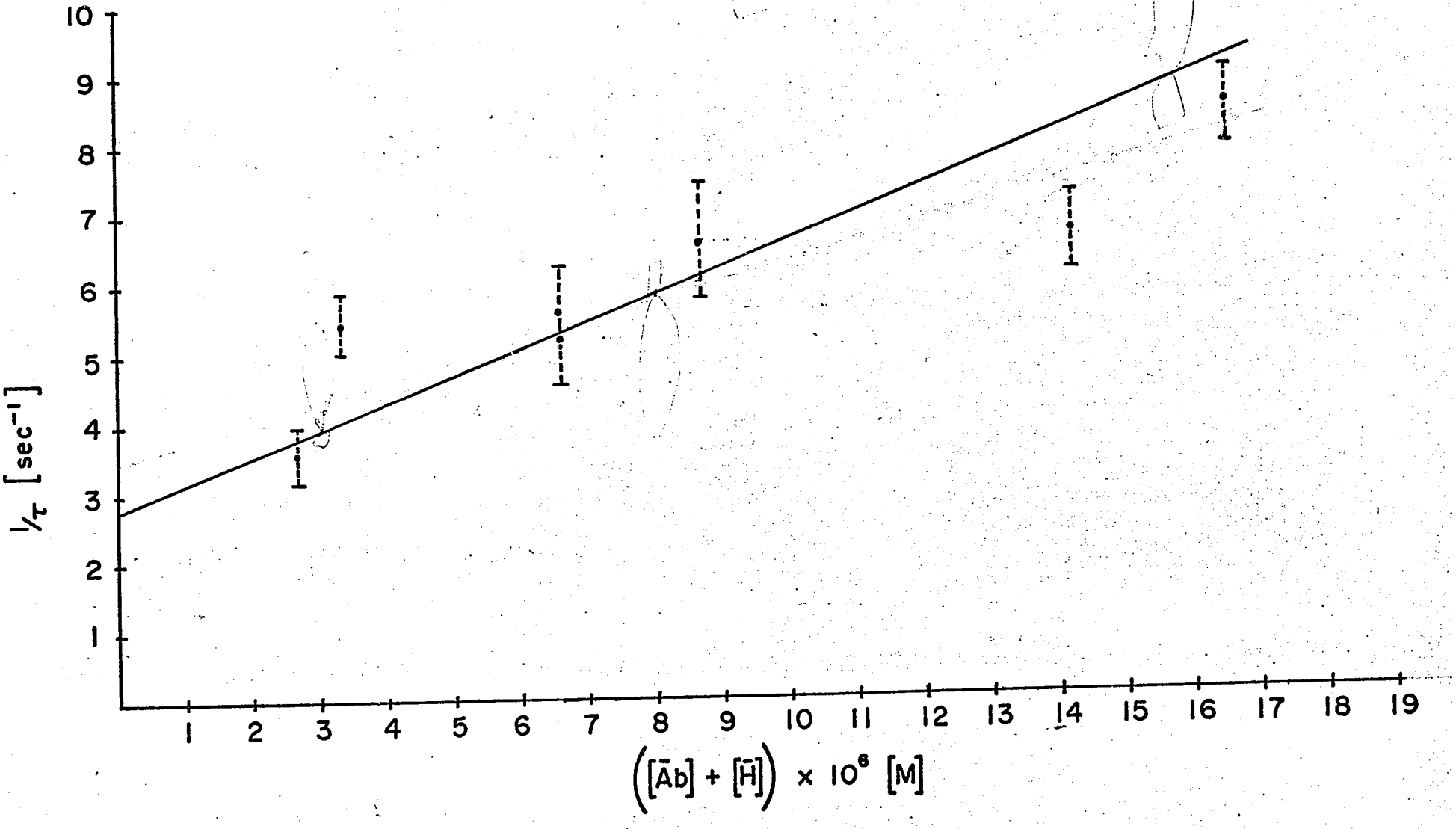
$$k_{12} = 3.7 \times 10^5 \ M^{-1} \ sec^{-1} \text{ and } k_{21} = 2.7 \ sec^{-1}.$$

It will be noted from Table XI that the relaxation times and, also, the rate constants, obtained in this system, differ considerably from those previously obtained in other similar antibody-hapten systems (120, 121). For example, relaxation times of a few milliseconds and forward rate constants of the order of $10^7 \ M^{-1} \ sec^{-1}$ have been obtained in the phenylarsonate system using antibody-hapten solutions of similar concentration to those used here (121). In order to obtain further information about the reaction in the IN-3, 6S-2DNP system, therefore, the effects buffer salt concentration, hapten structure and nature of γ-globulin preparation upon the reaction rate were investigated. The results obtained are described below.

(1) Temperature jump experiments were carried out with IN-3, 6S-4DNP- antibody solutions of varying concentrations at pH 4.5 where all free and bound dye present is in the protonated form (166). No relaxation effect was observed under these conditions, a result which

Figure 52

Concentration dependence of $1/\tau$ for the IN-3, 6S-2DNP ——— anti-DNP system at pH 8, μ = 0.1.


10
9
8
7
6
5
4
3
2
1
1/τ [sec⁻¹]
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
([Ab̄] + [H̄]) × 10⁶ [M]

could be due either to lack of sensitivity of the measurements or to the enthalpy of reaction being very small.

(II) Two different borate buffers of pH 8 μ= 0.1, were prepared. These differed in that in one the ionic strength due to the buffering salts was 0.025, while in the other it was 0.075. Identical amounts of antibody and hapten (IN-3, 6S-2DNP) were mixed in these two buffers ([AB] = 3.3×10^{-6} M, Hapten = 12×10^{-6} M). The visible spectra of the solutions under these two different conditions were identical indicating that the concentration of buffering salts had no obvious effect on the combination of hapten with antibody. Similarly, temperature jump experiments with these two solutions gave identical values for the relaxation time, i.e. τ = 1.06 sec.

(III) An attempt was made to synthesize the methylated naptholic derivative of IN-3, 6S-2DNP on the basis of the rationale that any color change observed upon combination of such a hapten with antibody could not be due to a protonation reaction. The hapten was therefore reacted with ketene, diazomethane and dimethyl sulfate; all these reagents are known as naptholic group methylating agents (192). Unfortunately, the products of these reactions were all found to have no visible absorption and it was concluded, therefore, that these reagents had destroyed the azo group of the original compound.

(IV) In order to investigate the possibility that the slow relaxation time observed was due to some property of the γ-globulin preparation used, experiments were also performed on another antibody preparation isolated from the serum of a different rabbit. The antibody concentration of this preparation was found by precipitin analysis to be

approximately 10% less than that of the preparation used above. Relaxation times obtained in exploratory experiments with this preparation were of the same order of magnitude as those given above.

The slow reaction in this system was also investigated using the Durrum-Gibson flow machine. Two sets of experiments were performed at pH 8 $\mu = 0.1$ using antibody solutions that were 5×10^{-6} M in antibody sites and hapten solutions containing 5×10^{-6} [Fig. 53] or 12×10^{-6} M IN-3, 6S-2DNP. A slow reaction was observed in both cases with half life times of approximately 0.4 and 0.3 seconds, respectively. Calculations of k_1 from Fig. 53 using the simplified expression that applies in the case of equal reactant concentrations

$$t_{1/2} = \frac{1}{k_1 a} \qquad [12]$$

where $t_{1/2}$ and a are the half life time and initial reactant concentrations, respectively, gave a value of $k_1 = 5 \times 10^5$ M^{-1} sec^{-1}, in good agreement with that previously determined from relaxation measurements.

C) Reaction of IN-2S-4DNP with Anti-DNP Antibody

Temperature jump experiments were carried out with this hapten at pH 9.5, $\mu = 0.1$. This high pH was used to ensure that all free dye present would be in the ionized form. No relaxation effects were observed in solutions of this hapten in buffer alone or in normal γ-globulin solutions of the same total protein concentrations as those containing anti-DNP antibody.

The concentrations of antibody and hapten in the solutions used for these experiments and the relaxation times calculated from the corresponding experiments are given in Table XII, while a representative

Figure 53

Example of a flow experiment at pH 8, μ = 0.1 in the IN-3, 6S-2DNP———anti-DNP system. Upper curve 480 mμ lower curve 600 mμ. Sweep 0.5 sec/cm. Horizontal lines are sweeps triggered after the system had reached equilibrium.

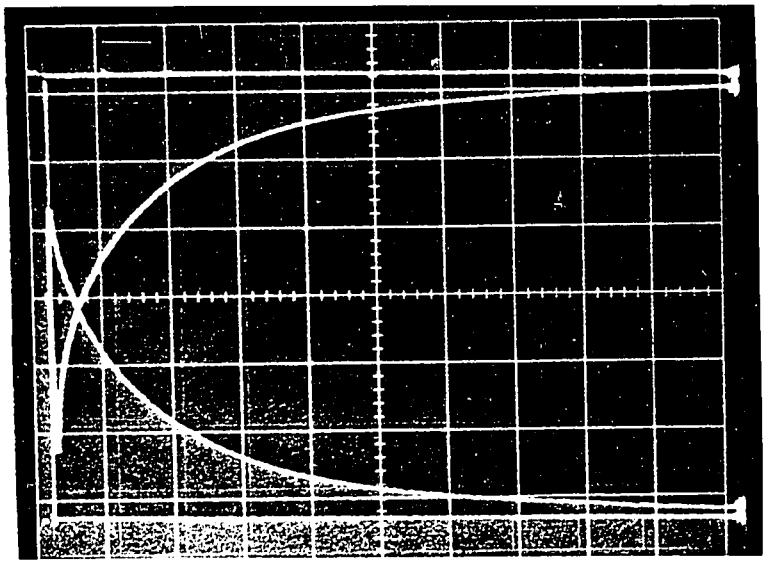


Table XII

Relaxation Experiments with the IN-2S-4DNP——Anti-DNP System pH 9.5

[ab] total $\times 10^6$ [M]	[H] total $\times 10^6$ [M]	[H] bound $\times 10^6$ [M]	$[\bar{Ab}]$ $\times 10^6$ [M]	$[\bar{H}]$ $\times 10^6$ [M]	$[(\bar{Ab})+(\bar{H})]$ $\times 10^6$ [M]	τ $\times 10^3$[sec]	$1/\tau$ $\times 10^{-3}$[sec^{-1}]
13.2	24.1	13.2	-	10.83	10.8	0.95	1.05
13.7	21.3	13.7	-	7.55	7.55	1.47	0.68
12.7	17.8	12.7	-	5.10	5.10	1.60	0.63
9.95	7.05	7.05	2.90	-	2.90	1.75	0.57
6.31	6.42	5.97	0.34	0.45	0.79	2.50	0.40

relaxation curve is shown in Fig. 54. The concentration dependence of $1/\tau$ for these experiments is plotted in Fig. 55 and the values for the forward and reverse rate constants, calculated on the assumption that the reaction is of the form

$$Ab + Hp \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AbH$$

were $k_{12} = 6.4 \times 10^7\ M^{-1}\ sec^{-1}$ and $k_{21} = 320\ sec^{-1}$

Preliminary experiments were also performed at pH 8 $\mu = 0.1$ with this system. Although no relaxation times were calculated, inspection of these results indicated that these were of the same magnitude as those found at pH 9.5.

DISCUSSION

Equilibrium Studies

In their study of the equilibrium aspects of the interaction of IN-3, 6S-2DNP with anti-DNP antibodies Metzger et al. (166) pointed out that the reaction of a hapten that can undergo an apparent change in pK upon binding to antibody can be represented in terms of the following reactions

$$Ab + DH \rightleftharpoons AbDH$$

$$Ab + D^- \rightleftharpoons AbD^-$$

$$AbD^- + H^+ \rightleftharpoons AbDH$$

where Ab represents an antibody site and D^- and DH are the ionized and protonated forms of the free hapten, respectively.

In any given system and at given concentrations of hapten and antibody the relative concentrations of the various species D^-, DH, AbD^- and AbDH will depend on the equilibrium constants for these reactions as well as on the pH of the solution. This was demonstrated spectrophoto-

Figure 54

Example of a relaxation curve in the 1N-2S-4DNP——— anti-DNP system at pH 9.5 μ = 0.1. Sweep - 1 milli. sec/cm

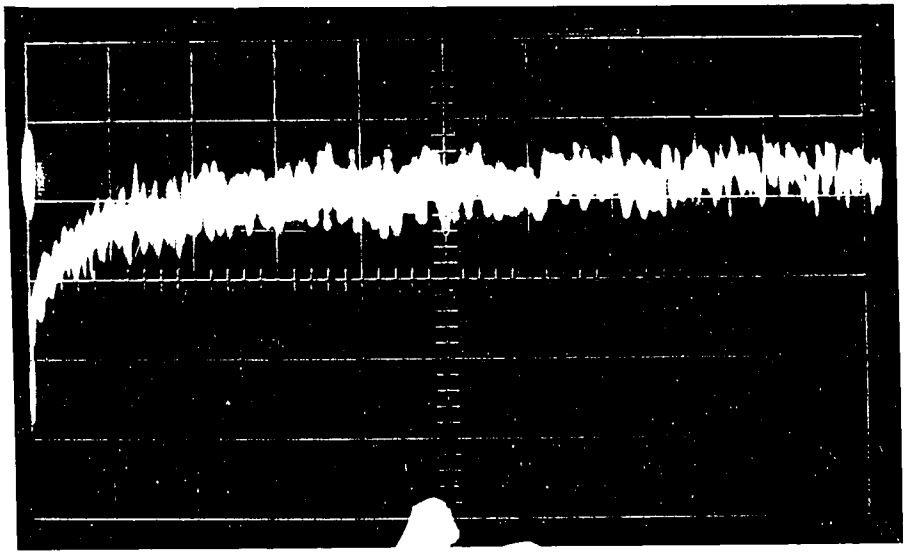
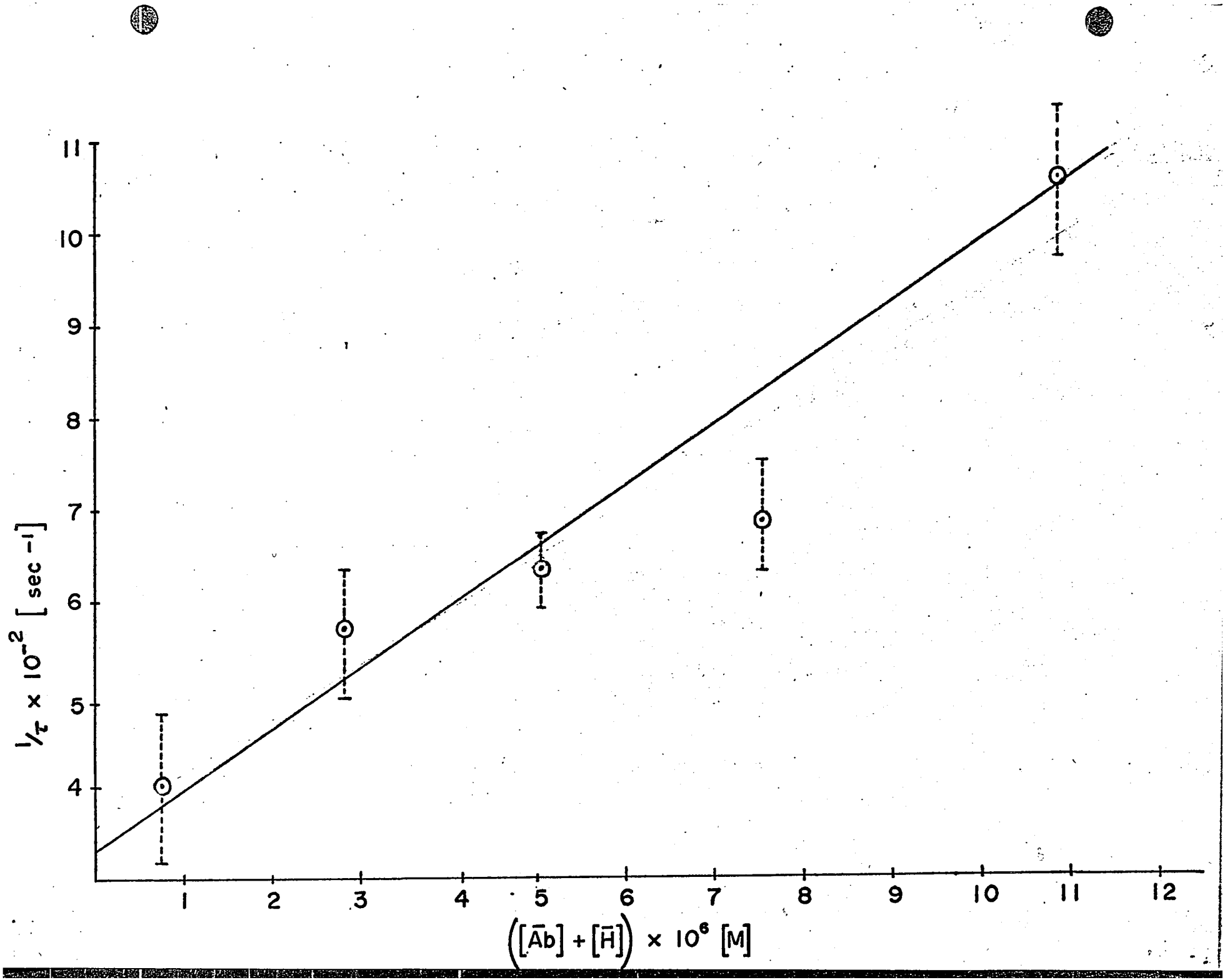


Figure 55

Concentration dependence of $1/\tau$ for the IN-2S-4DNP——— anti-DNP system at pH 9.5 $\mu = 0.1$.


11
10
9
8
7
6
5
4
1/τ × 10⁻² [sec⁻¹]
1
2
3
4
5
6
7
8
9
10
11
12
([Ab̄] + [H̄]) × 10⁶ [M]

metrically in the IN-3, 6S-2DNP system (166) and has also been shown to be true in the case of IN-2S-4DNP in this study. Thus, at pH 4.6 binding of IN-2S-4DNP to antibody produced only a slight change in the spectrum of the dye [Fig. 43]. At higher pH's, however, the effect of binding on the dye's spectrum was more obvious, as was seen in Figs. 43 and 45 for experiments done at pH 8 and 9.5 respectively.

The effects produced by antibody on the spectrum of IN-2S-4DNP at pH 8 and 9.5 differed considerably, i.e. at the higher pH the absorbance of the bound form of the hapten in the 480 mμ region decreased and a new peak at about 635 mμ appeared. The spectrum of the free dye also undergoes a change at this pH due to titration of the naptholic OH group which leads to an increase in absorption at ~615 mμ. However, by analogy to the IN-3, 6S-2DNP system and in view of the different positions of the absorption maxima, it seems logical to interpret the different spectra of antibody-hapten solutions at these two pH's in terms of ionization of the bound hapten. This conclusion is also confirmed by the presence of two isosbestic points in Fig. 45, indicating that at pH 9.5 three forms of the hapten IN-2S-4DNP are present. As all free dye at this pH must be in the ionized form, the remaining species are considered to be two forms of the bound hapten - in which the naptholic OH group is neutral or ionized.

Spectral binding measurements showed that both IN-2S-4DNP and IN-3, 6S-2DNP exhibited a high affinity for anti-DNP antibodies. The high value of K_o (10×10^6 M^{-1}) in the IN-2S-4DNP system at pH 9.5* does appear interesting in view of the finding that the affinity of

---

* It should be noted that values obtained for K_o refer to the overall binding of both forms of the bound hapten i.e. AbD^- and AbDH.

IN-3, 6S-2DNP for anti-DNP antibody began to exhibit a marked decrease in this pH region (166). This could be due to charge repulsion between the negatively charged hapten and the protein, which becomes increasingly more negative in this pK region (193). The absence of such an effect in the case of IN-2S-4DNP might be due to its possessing one less sulfonic acid group than IN-3, 6S-2DNP or to the different positions of the charged groups and haptenic determinant group in these two haptens.

In summing up the various observations made in the equilibrium studies of these systems, it appears that in spite of the differences in the structures of the three azo dyes used, i.e. IN-2S-4DNP, IN-2S-4pNP and IN-3, 6S-2DNP, all of these molecules exhibited similar spectral changes in the presence of rabbit anti-dinitrophenyl antibody. Thus, binding of each dye to antibody at pH 8 caused a shift in the spectrum of the (ionized, free) hapten to that corresponding to the free, protonated form of the dye. Moreover, the behavior of the IN-2S-4pNP system indicated that although this hapten lacked a nitro group in the 2 position, it interacted sufficiently with anti-dinitrophenyl antibody to exhibit a spectral change.

The latter result is to be expected in view of the extensive cross reactions of nitro- and dinitro- phenyl haptens (194). However, a consideration of the similar spectral effects caused by binding to antibody binding on IN-2S-4DNP and IN-3, 6S-2DNP would seem to indicate that the spectral behavior of these dye-hapten antibody systems is relatively insensitive to the overall structure of the hapten. Thus, these results might support the premise (166) that these spectral changes are due to a non-specific, secondary interaction which does not involve the haptenic

determinant group, such as a stabilization of the bound hapten in a protonated form because of the hydrophobic nature of the binding site.

Kinetic Studies

In accordance with the reaction schemes mentioned at the beginning of this discussion it is possible to represent the overall kinetic behavior of the hapten-antibody systems studied in terms of the following mechanism:

$$\begin{array}{ccccc} & \text{I} & & & \text{II} \\ \text{Ab} + \text{D}^- & & \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} & & \text{AbD}^- \\ \downarrow\uparrow & & & & \downarrow\uparrow \\ \text{Ab} + \text{DH} & & \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} & & \text{AbDH} \\ & \text{III} & & & \text{IV} \end{array}$$

where Ab, D^-, and DH are as previously described. One can assume that the equilibration steps between states I and III and II and IV are very fast relative to the other reactions in the system since under the experimental conditions of this study both these steps involve protolytic reactions in a buffered system. Moreover, if these reactions were slow then two relaxation times should have been observed, whereas no evidence of this was found. Keeping the above in mind and noting that.

$$\text{Ab} + \text{AbD}^- + \text{AbDH} = \text{Constant} = \text{total antibody} \qquad [13]$$

$$\text{D}^- + \text{DH} + \text{AbD}^- + \text{AbDH} = \text{Constant} = \text{total hapten} \qquad [14]$$

and defining $D_+ = D^- + DH$ as total FREE hapten [15]

$$K = \frac{[H^+][D^-]}{[DH]} \qquad [16]$$

and

$$K' = \frac{[H^+][AbD^-]}{[AbDH]} \qquad [17]$$

one can show that

$$1/\tau = k_{21}\left(\frac{K'}{K' + [H^+]}\right) + k_{43}\left(\frac{[H^+]}{[H^+] + K'}\right) + \left[k_{12}\frac{K}{K + [H^+]} + k_{34}\frac{[H^+]}{[H^+] + K}\right]\left(\bar{A}b + \bar{D}_+\right) \quad [18]$$

$$\text{or} \quad 1/\tau = k_{r\,app} + k_{f\,app}\left(\bar{A}b + \bar{D}_+\right) * \quad [19]$$

It is therefore obvious from equation [19] that the relaxation time, τ, for an overall reaction scheme such as the one mentioned, would show an apparently identical concentration dependence at a given pH to that expected for the simple process of

$$Ab + Hp \rightleftharpoons AbHp$$

In fact, however, the magnitude of the relaxation time will depend both on the concentrations of $\bar{A}b$ and $\bar{D}$ as well as on the pH of the solution and on the pK's of the bound and free forms of the hapten.

Two limiting forms of equation [18] may be considered:

(i) If $[H^+] \gg K \gg K'$ i.e. both free and bound forms of the dye are protonated

then $$1/\tau = k_{43} + k_{34}\left(\bar{A}b + \bar{D}_+\right) \quad [20]$$

(ii) If $[H^+] \ll K \ll K'$ i.e. both free and bound forms of the dye are in the deprotonated forms then

$$1/\tau = k_{21} + k_{12}\left(\bar{A}b + \bar{D}_+\right) \quad [21]$$

In view of the above it should in principle, be possible to determine all four rate constants by determining the concentration dependence of τ at various pH values.

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* A similar mechanism and behavior have been postulated by Dunford et al for the binding of fluoride ion by horse radish peroxidase (195). This equation has been derived in detail in Appendix A.

In the present study the rate constant for the association of anti-DNP antibody with IN-3, 6S-2DNP at pH 8 was found to be 3.7×10^5 M^{-1} sec^{-1}. This value was smaller by two orders of magnitude then those calculated for the association step in other antibody-hapten systems (119-121). Moreover, there was a significant discrepancy between the values calculated for K_o from equilibrium binding experiments and the ratio of k_{12} to k_{21}. One reason for this may be that the value of k_{12} calculated from the plot of $1/\tau$ vs $(\bar{A}b + \bar{D})$ in Fig. 52 was in fact a complex rate constant, $k_{f\ app}$, as defined in equation [18] and [19].

In view of this it is worthwhile to consider the possibility that at pH 8 the antibody reacts preferentially with the small amount of hapten that is present in the protonated form and therefore that the reaction involves only states I, III, IV of the reaction scheme mentioned. Such a process might be favored if, as mentioned previously, the ionized form of the hapten could not interact with the antibody site due to charge repulsion of a negative group on the protein or if water of hydration associated with the ionized naptholic OH group led to unfavourable steric interactions.* If this were true then the concentration dependence of $1/\tau$ in this system would be given by the relation

$$1/\tau = k_{43} + k_{34}\left(\frac{[H^+]}{[H^+] + K}\right)(\bar{A}b + \bar{D}_+) \quad ** \qquad [23]$$

The slope of Fig. 52, i.e. 3.7×10^5 M^{-1} sec^{-1}, would then equal

$$k_{34}\left(\frac{[H^+]}{[H^+] + K}\right)$$

which after substitution of the appropriate values of $[H^+]$ and K, i.e.

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* The steric effect of water of hydration has been noted in the extensive cross reaction of substituted benzene haptens e.g. phenyl iodide, with antibodies directed against pyridine (95).

** Please see Appendix B

10^{-8} and 3.2×10^{7} (166) respectively, leads to a value of 1.2×10^{7} M^{-1} sec^{-1} for k_{34}, and a calculated K_o value of 4.2×10^{6} M^{-1}.* This value for k_{34} is in agreement with those obtained for association rate constants in other antibody-hapten systems and also agrees quite well with that found by Day et al, i.e. 8×10^{7} M^{-1} sec^{-1}, in fluorescence quenching, stopped flow experiments with this system at pH 5 (166), at which pH almost all of the bound and free forms of the hapten are present in their protonated forms. Moreover, this mechanism leads to satisfactory agreement between values of K_o calculated from kinetic and equilibrium data.

It should be stressed that the above interpretation is not unequivocal and obviously can only be verified by a complete study of the pH dependence of the reaction. This was attempted to a certain extent in this study. However, as has been mentioned, experiments at pH 4.5 proved unsuccessful, while lack of sufficient antibody prevented further experimentation at high pH. In any case the latter might have proven difficult to perform in view of the dissociation of antibody-hapten complexes in this system at pH's greater than 9.5 (166). This latter complication would of course invalidate the simple mechanism proposed as it does not take into account any variation in rate constants with pH due to charge repulsion effects.

In the case of IN-2S-4DNP the value found for k_f was calculated as 6.4×10^{7} M^{-1} sec^{-1}. The agreement of this result with that found by

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*It should be noted that the alternate assumption that the reaction involves states I, II and IV, leads to values of k_{12} and k_{21} of 3.7×10^{5} M^{-1} sec^{-1} (unchanged from that mentioned previously) and 30 sec^{-1}, respectively.

other authors would indicate that in this system the reaction involves states I, II and IV of the proposed mechanism.* The different behavior of this hapten from IN-3, 6S-2DNP could be explained in terms of the different orientations of the naptholic OH and DNP groups in these two dyes. In the case of IN-2S-4DNP any steric hindrance of water molecules about the naptholic O^- group would not interfere with interaction of the DNP determinant with the antibody binding site; moreover, as previously mentioned the lower (negative) charge of this hapten would reduce the effect of repulsion by the (negatively) charged protein.

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* No satisfactory explanation can be offered at present for the discrepancy between the equilibrium constant calculated for this system by static and kinetic experiments.

GENERAL DISCUSSION

As has been stated throughout this thesis the nature of the interactions that lead to the spectral shifts or apparent pK changes of pH indicators upon binding to proteins are poorly understood. Moreover, as these phenomena have been employed as an analytical tool for the study of dye-protein interactions in both this and other studies, it was felt that an intensive investigation of these reactions would prove worthwhile. As relevant to one aspect of these phenomena, a comparison of binding data for antibody-dye hapten interactions, as obtained by spectral and equilibrium dialysis techniques, was carried out. It was pointed out that "a priori" reasoning might lead one to expect that a dye-hapten would not possess a unique, single-valued, bound extinction coefficient in the presence of an antibody population that exhibited a distribution of affinities for it. On the other hand it could also be possible that the dependence of the extinction coefficient of the bound form of the hapten on the variations in antibody-hapten interactions that lead to heterogeneity would be undetectable experimentally. In such a case the bound extinction coefficient could be considered, for all practical purposes, to be single valued.

In the experiments reported in this study with anti-dinitrophenyl antibodies and IN-3, 6S-2DNP, a small difference was noted between the binding curves calculated from spectrophotometric and equilibrium dialysis measurements. Because of the small magnitude of this difference, $\sim$10%, it was felt that, in this particular system, valid results could be obtained with the spectrophotometric method. However, this conclusion cannot be applied

indiscriminately to all antibody-hapten systems. For example it was found (196) that antibodies produced in different rabbits against the same determinant group, p-arsanilate, caused different spectral changes in the same p-arsanilate azo-dye. This result could only be due to a variation in extinction coefficient of the dye on binding to the different molecules in an antibody population. It is felt, therefore, that in view of these results and of the above reasoning, that spectrophotometric data should be compared to those obtained by an absolute method, such as equilibrium dialysis, whenever possible.

The behavior of a dye exhibiting a spectral shift upon binding "non-specifically"* to a protein was explored in some detail in the study of the reaction of IN-2S-4DNP with BSA. Unfortunately, however, no unequivocal description of the mechanism of this spectral shift could be presented. Thus, although evidence was obtained supporting the idea that the spectral change was due to an interaction of the bound dye with carboxylate groups of the protein, it was noted that this interpretation might have been based on a fortuituous correspondence of the titration curve of the bound dye to that of the protein's carboxylate groups. In the same way it was not possible to distinguish between specific and non-specific effects due to the addition of anions which acted as inhibitors of the dye's spectral shift. Such difficulties often arise in the interpretation of reactions between proteins and small ligand molecules. In a molecule such as BSA, the complexities introduced because of its having a high molecular weight and large number of titrable groups often

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* Specific interactions usually refer to the reactions of enzymes with their substates, or of antibodies with their homologous haptens or antigens.

make it impossible to distinguish between or delineate the different effects produced by the alteration of a single external parameter. For example titration of the protein not only changes its charge, but can also lead to alterations in its structure, and all such effects must be considered in a detailed description of the mechanisms of reactions involving a protein. In view of such complications it was felt that a clearer and more complete understanding of the phenomena observed in this study could be obtained through recourse to indirect methods, rather than relying on observations based on the results of alterations in the molecule itself or in its environment.

An attempt was made to study the kinetics of the reaction of IN-2S-4DNP with BSA at pH 5 with the temperature-jump technique. Unfortunately, no relaxation effect was observed in these experiments; this may be interpreted as being due to the enthalpy of reaction being zero or very small in this system. Since the apparatus became available just prior to the termination of this study only one (trial) experiment was performed with this system using the Durrum-Gibson stopped flow instrument. At reactant concentrations of about 5×10^{-6} M (dye and protein) the system exhibited a large signal change with a half life of about 10 milliseconds, indicating that the reaction could be studied by this technique.

A comparison of the results of the equilibrium studies performed with BSA and antibodies, with IN-2S-4DNP, points to the differences between specific and non-specific protein-ligand reactions. These two systems differed most obviously in their equilibrium constants, that for the combination of antibody with IN-2S-4DNP being 100 times greater than that

of the BSA system. It should be noted that a strict comparison of these two systems cannot be made as experiments were performed at pH 5 with BSA and at pH 9.5 with antibodies. However this comparison is still felt to be valid as it would be expected that the affinity of BSA for the dye would be less at the higher pH due to increased change repulsion.

In view of the fact that no study was made of the relative affinities of the DNP determinant group itself for the two proteins, it should be noted that the results obtained are indicative only of the strengths of the overall protein-dye bonds formed. Metzger et al (166) have pointed out that the contribution of a large molecular group, such as the 1-napthol, 2-sulfonic acid group, to the free energy of a hapten-antibody reaction can be quite considerable. As no inhibition experiments were performed with the 1N-2S-4DNP ——— antibody system, no comments can be made about the relevance of this argument to the results reported. However, it was seen that a 10,000 fold excess of the 1-napthol, 2-sulfonic acid did fail to displace the dye from its binding sites on the BSA molecule. In this system, therefore, it is obvious that the main contribution to the free energy of interaction is made by the 2, 4-dinitrophenyl group.

One apparently anomalous result was obtained in these equilibrium studies. Thus, although both 1N-2S-4DNP and 1N-2S-4pNP underwent similar bathochromic shifts in the presence of antibody at pH 8, only 1N-2S-4DNP exhibited a spectral shift in the presence of albumin at pH 5. This result indicates that in this case, the BSA molecule, in contrast to anti-DNP antibodies, can differentiate between two dye-haptens of similar structure. Although this conclusion might be interpreted in terms of a greater

specificity of the reactions in the dye-BSA system it is of course only relevant to those protein-dye interactions which lead to a spectral shift and does not necessarily reflect the relative affinities of the two proteins for the two dyes. The latter is obvious from the observation that no spectral shift was exhibited by IN-2S-4DNP at pH 2 in the presence of BSA even though all of the dye present was bound to the protein.

Further comparisons of the different effects of BSA and antibodies on the spectra of the dyes used, also point to obvious differences in the binding sites of the two proteins. Thus, for example, at pH 5 and at the same conditions of ionic strength [Fig. 46], albumin produced a large spectral shift in IN-2S-4DNP, while the antibody had almost no effect. The reverse was found to be true at pH 9.5 [Figs. 8, 45], as under these conditions no large spectral shift was observed in the presence of albumin. Although these differences do exist between the two proteins, it is possible by analogy to the hypothesis advanced in Chapter II, that the spectral changes of these dye haptens in the presence of antibody are due to similar types of interactions i.e. between the bound hapten and a positive group e.g. the ϵ-amino group of lysine. The formation of a strong hydrogen bond, or actual hydrogen ion transfer, by such a proton donating group would then allow the bound hapten to exist in a "protonated" form on the protein with the result that a spectral shift would be observed. Moreover, the pK of the lysine group is about 10 (193), which is in the region of that of the bound form of IN-3, 6S-2DNP (166); the spectral results obtained here also indicate that the titration of bound IN-2S-4DNP occurs in this pH region.

As an alternative to this hypothesis, Metzger et al (166) suggested that the spectral shift of IN-3, 6S-2DNP was due to the binding of this hapten in a hydrophobic region of the antibody molecule. In such an area the protonated OH form of the hapten would be more stable and in addition could be stabilized, intramolecularly, through hydrogen bonding to either the azo or 2-nitro groups of the hapten. Obviously, the same type of process could occur with IN-2S-4DNP or IN-2S-4pNP, as in both these haptens stabilization of the protonated, bound form of the hapten could be accomplished through hydrogen bonding to the oxygen atoms of the 2-sulfonic acid group.

The comparative study of the kinetics of the reaction of IN-2S-4DNP and IN-3, 6S-2DNP with antidinitrophenyl antibodies was also performed to gain further information about antibody dye reactions, leading to spectral shifts. It was found that in spite of their having different charge properties and structures both dyes exhibited similar spectral shifts in the presence of antibodies. A difference was noted in the affinity of the two molecules for the antibody and the higher binding of IN-2S-4DNP was suggested to be due to its lower negative charge and to the greater distance between the naptholic OH group and the DNP determinant group in this dye.

Much larger differences were noted between these two haptens in kinetic studies. The rate constants calculated from experiments with IN-2S-4DNP were found to be of the same order of magnitude as those calculated for other hapten-antibody systems. The behavior of IN-3, 6S-2DNP, however, was found to be unusual in that relatively low values for the forward rate constant were obtained. The different results led

to an interpretation of the reaction mechanism of IN-3, 6S-2DNP with antibody at pH 8 in terms of a preferred reaction of the protein with the small amount of protonated free dye in solution. On the other hand, the reaction of IN-2S-4DNP appeared to involve the more obvious step of reaction of the ionized form of the free dye with the antibody site. These differences were explained in terms of the different structures of the two haptens and a possible inhibitory steric effect of bound water molecules associated with the ionized form of the naptholic OH group in IN-3, 6S-2DNP. These differences could also be related to the interactions that lead to the dye's spectral shift on binding to antibody. Thus in the case of IN-3, 6S-2DNP it appears, as Metzger et al have suggested (166), that the hapten acquires a proton from solution and is stabilized on the antibody in a protonated form because of the hydrophobic milieu of the antibody site. In the case of IN-2S-4DNP, the antibody appears to react with the ionized form of the hapten. In this case then, the proton acquired by the bound dye could be donated from the solution, or as postulated earlier in this discussion, from a donor group on the protein.

Theoretical considerations of the kinetics of protein-ligand interactions have led to the calculation of a value of 1.5×10^9 M^{-1} sec^{-1} for the forward rate constant of a diffusion controlled reaction between uncharged reactants having spherical symmetry (197). The value obtained for this rate constant in the IN-2S-4DNP system, 6.4×10^7 M^{-1} sec^{-1}, is substantially less than the above. The difference between these two values could be due, as has been suggested by other authors (119, 120), to the necessity of overcoming repulsive forces between the similarly

charged hapten and antibody molecules. It is also possible that a conformational change is involved in the primary antibody-hapten combination step, as well as in the processes leading to a spectral shift. Both these factors would lead to an increase in the activation energy of the reaction and, therefore, to smaller forward rate constants than the theoretical maximum mentioned above.

It should be noted that although the antibody preparation appeared to be markedly heterogeneous with respect to its equilibrium constant, as seen from the curvature of $1/_b$ vs $1/_c$ plots, this was not obvious from the kinetic behavior of these systems; antibody heterogeneity has also not been observed directly in the kinetic experiments in other temperature-jump relaxation studies of these reactions (120, 121). By contrast antibody heterogeneity has been detected directly in fluorescence stopped flow measurements of these systems performed by other authors (119). The above points to one disadvantage of the temperature-jump technique, i.e. that the sensitivity of this method is quite low, due to the fact that the effects measured are usually small. By comparison, the signals measured in flow experiments are relatively much larger, as they may represent the difference in absorption of solutions in which the hapten is all in the free state or all bound.

Another difficulty encountered in this work was due to a limitation of the temperature-jump apparatus employed. Thus, in order to allow observation of the extent of a hapten-antibody reaction, the hapten employed had to undergo a change in its visible spectrum upon reaction with antibody. Six dye haptens, of different structure, containing the phenyl arsonic acid, or p-phenyl azo phenyl arsonic acid

determinant group* were synthesized, but unfortunately were not found to undergo any detectable visible spectral shift in the presence of homologous antibodies. In fact to this date only eight dye haptens (known to the author) have been found to exhibit this property. In view of this, it is obvious that a modification of the apparatus to allow measurements of systems exhibiting fluorescence changes would greatly increase the versatility of this technique. In particular such a modification would allow verification of the proposed mechanism for the reaction of IN-3, 6S-2DNP with antibody. Moreover, as the energy transfer processes involved in fluorescence quenching are over within 10^{-12} to 10^{-13} of a second (197), observations of the extent of reaction using this technique would be independent of the rates of any protonation reactions or conformational changes occurring in these systems, assuming of course that the latter had no effect on the quenching step.

* The different haptens were prepared by the coupling of the diazonium salts of 4-amino benzene arsonic acid and 4-(4-amino phenylazo) - benzene arsonic acid to l-napthol 4-sulfonic acid, l-napthol 3, 6-disulfonic acid and 2-napthol 3, 6-disulfonic acid respectively.

Summary

1) The interaction of the dyes IN-2S-4DNP, IN-2S-4pNP and IN-3, 6S-2DNP with BSA was shown to lead to large spectral shifts under appropriate conditions.

2) The equilibrium constant for the reaction of IN-2S-4DNP with BSA was found to be about 2×10^5 at pH 5, μ=0.02. The pK of the bound form of this dye was determined and the effect of anions on the titration curve of the dye evaluated.

3) The effect of pH, of structurally related inhibitors and of the dielectric constant and ionic strength of the medium on the reaction was evaluated. Two types of inhibition i.e. A) displacement of the dye from the protein and B) reduction of the size or extent of the spectral shift without displacement of the dye, were found to exist and were explained on the basis of protein denaturation and electrostatic effects, respectively.

4) The spectral shift of the dye on binding was postulated to involve the formation of "hydrogen" bonds with carboxylate groups of the protein. This conclusion was based on the results of added anions on the dye's titration curve and on the correspondence of the titration curve to that of the protein's carboxylate groups.

5) A comparison of the values of equilibrium constants determined by spectrophotometric and equilibrium dialysis measurements in the IN-3, 6S-2DNP _____ antibody system showed that the former technique was valid in this case.

6) Difficulties in interpreting the results of spectrophotometric binding measurements due to the heterogeneity of antibodies with respect to their binding affinities were discussed.

7) The effect of anti-DNP antibodies on the spectra of IN-2S-4DNP, IN-2S-4pNP and IN-3, 6S-2DNP was determined. It was found that these three dye-haptens exhibited similar spectral changes on binding to antibody, which at pH 8 could be interpreted in terms of a stabilization of the bound molecule in a protonated form.

8) The kinetics of the reactions of IN-2S-4DNP and IN-3, 6S-2DNP with anti-DNP antibodies was investigated. The IN-2S-4DNP system exhibited similar behavior to that of previous systems studied and the rate constant for the combination of antibody with hapten was evaluated as $6.4 \times 10^7\ M^{-1}\ sec^{-1}$.

9) The apparent forward rate constant in the IN-3, 6S-2DNP system was found to be lower by two orders of magnitude than the above. This difference in behavior was postulated to be due to structural dissimilarities in the two haptens which led to a preferred reaction of the anti-DNP antibody, at this pH, with the protonated form of IN-3, 6S-2DNP.

Claims to Originality

1) The interaction of IN-2S-4DNP and IN-3, 6S-2DNP with BSA was observed spectrophotometrically and the equilibrium constant for the reaction of the former dye with the protein determined.

2) The effects of pH, dielectric constant, etc., and various inhibitors on the reaction of IN-2S-4DNP with BSA were evaluated. The pK of the bound form of the dye was determined and the effects of anions on the bound dye's titration curve studied.

3) A possible explanation for the mechanism of the spectral shift was advanced in terms of interaction between bound dye molecules and carboxylate groups of the protein.

4) A comparison was made of spectrophotometric and equilibrium dialysis techniques for the evaluation of equilibrium constants in antibody-hapten systems.

5) The possible effects of antibody heterogeneity on results of such spectrophotometric experiments were discussed and it was suggested that reference be made to absolute methods when interpreting these results.

6) The interaction of IN-2S-4DNP with anti-DNP antibodies was investigated and the large spectral shift of this hapten in the presence of antibody used for evaluation of the equilibrium constant for this reaction.

7) The equilibrium constant for the reaction of IN-2S-4DNP with antibody was found to be 1×10^7 in spite of the high pH, 9.5, at which experiments were performed.

8) A comparison of the kinetics of the reactions of IN-2S-4DNP and IN-3, 6S-2DNP with anti-DNP antibodies was made using the temperature-jump technique.

9) A slow reaction was observed in the IN-3, 6S-2DNP system at pH 8. The suggestion was advanced that this was due to a preferred reaction of the antibody with the protonated form of the free dye-hapten.

10) The rate constants for the association and dissociation steps, for the reaction of IN-2S-4DNP with antibody, were found to be 6.4×10^7 M^{-1} sec^{-1} and 320 sec^{-1} respectively.

11) The difference in kinetic behavior of IN-3, 6S-2DNP and IN-2S-4DNP was attributed to structural and charge differences between the two dye-haptens. It was also suggested that the type of interactions that lead to spectral shifts in these systems might depend on the structure of the particular dye-hapten involved.

APPENDIX A

For the system

$$\begin{array}{ccc} \text{I} & & \text{II} \\ Ab + D^- & \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} & AbD^- \\ \Updownarrow & & \Updownarrow \\ Ab + DH & \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} & AbDH \\ \text{III} & & \text{IV} \end{array}$$

$$\frac{d(Ab)}{dt} = - k_{12}\,(Ab)(D^-) - k_{34}\,(Ab)(DH) + k_{21}\,(AbD^-) + k_{43}\,(AbDH) \qquad (1)$$

Now $Ab + AbD^- + AbDH = \text{const.}$ (2)

$$DH + D^- + AbD^- + AbDH = \text{const.} \qquad (3)$$

Defining

$$K = \frac{(D^-)(H^+)}{DH} \qquad (4)$$

$$K^1 = \frac{(AbD^-)(H^+)}{(AbDH)} \qquad (5)$$

and

$$Ab = Ab + \Delta Ab \qquad (6)$$

$$D^- = \bar{D}^- + \Delta D^- \qquad (7)$$

$$AbD^- = AbD^- + \Delta AbD^- \qquad (8)$$

$$DH = DH + \Delta DH \qquad (9)$$

$$AbDH = AbDH + \Delta AbDH \qquad (10)$$

and substituting (6) to (10) into (1) (and neglecting small terms) gives

$$\frac{d\,\Delta Ab}{dt} = - k_{12}\,(\bar{D}^-)(\Delta Ab) - k_{12}\,(\bar{Ab})(\Delta D^-) - k_{34}\,(\overline{DH})(\Delta Ab) - k_{34}\,(\bar{Ab})(\Delta DH) + k_{21}\,(\Delta AbD^-) + k_{43}\,(\Delta AbDH) \qquad (11)$$

Now from (2) and (5)

$$Ab + (AbD^-)\left(\frac{K^1 + (H^+)}{K^1}\right) = \text{const.} \qquad (12)$$

$$\therefore \quad \Delta Ab + (\Delta AbD^-)\left(\frac{K^1 + (H^+)}{K^1}\right) = 0 \qquad (13)$$

and from (3), (4) and (5)

$$D^- \left(\frac{(H^+) + K}{K}\right) + (AbD^-)\left(\frac{K^1 + (H^+)}{K^1}\right) = \text{const.} \qquad (14)$$

$$\therefore \Delta D^- \left(\frac{(H^+) + K}{K}\right) + (\Delta AbD^-)\left(\frac{K^1 + (H^+)}{K^1}\right) = 0 \qquad (15)$$

Combining (13) and (16)

$$\Delta Ab = \Delta D^- \left(\frac{(H^+) + K}{K}\right) \qquad (16)$$

Also since

$$\Delta DH = \Delta Ab \left(\frac{(H^+)}{K + (H^+)}\right) \qquad (17)$$

$$\text{and also } \Delta Ab = -\Delta AbDH \left(\frac{(H^+) + K^1}{(H^+)}\right) \qquad (18)$$

Substitution of (13), (16), (17) and (18) into (11) and collecting terms leads to

$$\frac{d\ \Delta Ab}{dt} = -\Delta Ab \left[k_{12}\,\bar{D}^- + k_{34}\,\overline{DH} + k_{12}\,\overline{Ab}\left(\frac{K}{K + (H^+)}\right) + k_{34}\,\overline{Ab}\left(\frac{(H^+)}{K + (H^+)}\right)\right] - \Delta AB \left[k_{21}\left(\frac{K^1}{K^1 + (H^+)}\right) + k_{43}\left(\frac{(H+)}{K^1 + (H^+)}\right)\right] \qquad (19)$$

From Appendix B and as previously defined

$$\bar{D}_+ = \bar{D}^- + \overline{DH} \qquad (20)$$

and

$$\bar{D}^- = \frac{K}{K + (H^+)}\ \bar{D}_+ \qquad (21)$$

and

$$\overline{DH} = \left(\frac{(H^+)}{(H^+) + K}\right)\bar{D}_+ \qquad (22)$$

Therefore (19) yields

$$\frac{d\ \Delta Ab}{dt} = -\left[k_{12}\left(\frac{K}{K + (H^+)}\right) + k_{34}\left(\frac{(H^+)}{K + (H+)}\right)\right]\Delta Ab\ (\overline{Ab} + \bar{D}_+) - \left[k_{21}\left(\frac{K^1}{K^1 + (H^+)}\right) + k_{43}\left(\frac{(H^+)}{K^1 + (H^+)}\right)\right]\Delta Ab \qquad (23)$$

From this

$$1/\tau = k_{21}\left(\frac{K'}{K' + (H^+)}\right) + k_{43}\left(\frac{(H^+)}{K' + (H^+)}\right) + \left[k_{12}\left(\frac{K}{K + (H^+)}\right) + k_{34}\left(\frac{(H^+)}{(H^+) + K}\right)\right](\bar{A}b + \bar{D}_+) \qquad (24)$$

$$\therefore \quad 1/\tau = k_{r\ app} + k_{f\ app}\ (\bar{A}b + \bar{D}_+)$$

APPENDIX B

Considering a reaction involving states I, III and IV (state II may be neglected because of the relative values of the pH 8, and the pK of the conversion step from states II to IV, 9.5 (166), we can write the system as

$$\begin{array}{ccccc} Ab & + & DH & \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} & AbDH \\ & & \rotatebox{90}{$\rightleftharpoons$} & & \\ H^+ & + & D^- & & \end{array}$$

then $$d\frac{Ab}{dt} = -k_{12}(Ab)(DH) + k_{21}(AbDH) \qquad (1)$$

Defining

$$Ab = \bar{A}b + \Delta Ab \qquad (2)$$

$$DH = \bar{D}H + \Delta DH \qquad (3)$$

$$AbDH = AbDH + \Delta AbDH \qquad (4)$$

(where all terms are as previously defined and represent appropriate reactant concentrations).

and substituting 2-4 into 1 while neglecting small term products leads to

$$\frac{d\Delta Ab}{dt} = -k_{12}(\bar{A}b)(\Delta DH) - k_{12}(\bar{D}H)(\Delta Ab) - k_{21}(\Delta Ab\bar{D}H)$$

$$= -k_{12}(\bar{A}b)(\Delta DH) - k_{12}(\bar{D}H)(\Delta Ab) - k_{21}(\Delta Ab) \qquad (5)$$

Defining $$D_+ = D^- + DH \qquad (6)$$

and $$K = \frac{[D^-][H^+]}{[DH]} \qquad (7)$$

then $$D_+ = \frac{DH}{[H^+]}K + DH \qquad (8)$$

$$= DH\left(1 + \frac{K}{[H^+]}\right)$$

$$= DH\,\frac{(K + [H^+])}{[H^+]} \qquad (9)$$

Also since

$$\Delta D_+ = \Delta D^- + \Delta DH = \Delta Ab \qquad (10)$$

then $$\Delta Ab = \Delta DH \frac{(K + [H^+])}{[H^+]}$$

or $$\Delta DH = \Delta Ab \left(\frac{[H^+]}{K + [H^+]}\right) \quad (11)$$

Substituting into (5) above.

$$\frac{d\Delta Ab}{dt} = -k_{12}\left(\frac{[H^+]}{K + [H^+]}\right) \cdot \Delta Ab\,(\bar{A}b + \bar{D}_t) - k_{21}\Delta Ab \quad (12)$$

and therefore $$1/\tau = k_{21} + k_{12}\frac{[H^+]}{K + [H^+]}(\bar{A}b + \bar{D}_t) \quad (13)$$

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