ANTIBODY-HAPTEN AND PROTEIN-DYE INTERACTIONS

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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CHAPTER I

NATURE AND PROPERTIES OF ANTIBODIES AND ANTIGENS

According to the historical survey of immunology by Grabar (1) the "art" of immunization can be traced to ancient times. It was known for a long time that after exposure and recovery from an infectious disease one usually did not contract it again. In 1798 Jenner performed the first successful immunization against smallpox and his vaccination procedure is still used today. Kraus (2) observed that resistance could be developed not only by exposure to the intact bacteria, but also to their extracts or toxins.

Later it was shown that the introduction of a foreign substance into an animal induced the formation of globular serum proteins, which possessed the unique property of reacting specifically with the foreign substance. These globular proteins are known as antibodies and the foreign substances which elicit their production are known as antigens.

PROPERTIES OF ANTIGENS

All antigens have been shown to be macromolecules, most of them of biological origin, such as: blood cells, serum proteins, tissue extracts, conjugated proteins (3), polysaccharides (4) and lipids (5). More recently synthetic polymers such as polyvinyl-pyrrolidone (6) and polypeptides (7, 8) have been shown to possess antigenicity.

Landsteiner (9) made the fundamental discovery that antigenicity could be conferred by chemically well-defined groups, such as arsanilic acid and sulphanilic acid, provided that they were coupled to proteins by covalent bonds. These small molecules were called "haptens" by Landsteiner (from the Greek: hapten = to bind). By contrast, the injection of the uncoupled haptens does not stimulate antibody production.

Coupling of a large number of hapten molecules to a protein may completely change the specificity of the native protein. Thus, antibodies to iodinated horse serum will react with other iodinated proteins, but not with native horse serum (10). Similarly, Haurowitz et al (11) observed that when more than one hundred phenyl arsonate groups are introduced per molecule of horse gamma globulin, the antigenic specificity to the native globulin was completely abolished.

PROPERTIES OF ANTIBODIES

With regard to their electrophoretic properties, antibodies are mainly classed as serum gamma globulins (12), but there are some found also in the alpha and beta globulin fractions (13, 14). Antibodies (primarily those produced by rabbits) have been shown to be identical, with respect to their molecular weight, to normal globulins which have a molecular weight of about 160,000 (15, 16) and a sedimentation coefficient of 6.5 Svedberg units. Antibodies with molecular weights of the order of about 1,000,000 have been detected

in sera of some other species (17). It should be pointed out, however, that gamma globulins are not electrophoretically homogeneous as demonstrated, for example, by the reversible boundary-spreading phenomenon (18). Using electrophoresis convection, gamma globulins were shown to consist of several fractions (19). The only property which distinguishes antibodies from normal serum globulins, is their capacity to combine specifically with the antigen responsible for their formation.

Combining Sites

An antibody molecule is thought to differ from a normal globulin molecule by possessing areas which are configurationally complementary to the antigenic groupings with which they combine. Pauling (20) assumed that all antibody molecules possessed the same polypeptide chains as normal globulins and differed only in the configuration of these chains, and that an antibody molecule had at most two active sites. Furthermore, he postulated that the observed versatility of antibodies with respect to their complementariness to different antigens could be explained by the existence of an extremely large number of accessible configurations with nearly the same energy for the end parts of the globulin polypeptide chain. He also postulated that the central backbone portion of the antibody molecule could assume only one possible configuration characteristic of normal globulins, which would account for the antigenic similarity of antibodies and normal globulins. Pauling's hypothesis

that an antibody molecule has two combining sites was verified several years later by a number of investigators (21, 22, 23, 24).

Recently, it was shown that digestion of rabbit antibodies with crystalline papain in the presence of cysteine resulted in their degradation into three fragments, which were separable by chromatography on carboxymethyl cellulose (25, 26, 27). These fragments represented over 90 percent of the original protein and were non-dialyzable through Visking tubing. Two of these fragments (Fractions I and II) appeared to be almost identical in chemical, physical and immunochemical properties and had a molecular weight of about 50,000. Fraction III had a molecular weight of about 80,000. The relative yields of the three fractions were found to be almost identical and, therefore, it seemed likely that rabbit gamma globulin consisted of two identical portions joined to a third portion of quite different structure. It was demonstrated by Nisonoff et al (27, 28) that each of the fragments I and II of a rabbit antihapten antibody was univalent. The same authors (28, 29) also showed that gamma globulins can be degraded by the combined or successive actions of pepsin and reducing agents (such as cysteine, thioglycolate, and 2-mercaptoethylamine) into fragments essentially similar to those obtained by papain digestion. Treatment with pepsin alone caused the reduction in the molecular weight of the antibody molecule from 160,000 to 106,000 (30). The latter fragment was, however, still bivalent, and

on subsequent treatment with one of the reducing agents, capable of splitting disulfide bonds, was degraded into univalent fragments with a molecular weight of about 56,000. Thus, it could be conceived that an antibody molecule consists of two identical fragments joined through a disulfide bond, each having one antibody combining site, and that a third fragment is linked to one or both of these fragments through covalent bonds.

The combining site makes up a relatively small portion of the antibody molecule, and its upper limit has been found for human anti-dextran antibodies, to be an area complementary to an open chain of about 6-7 glucose units (31, 32, 33). The dimensions of the hexasaccharide group are about 34 x 12 x 7 A. Great efforts have been made to elucidate the factors determining the molecular complementariness of antigenic and antibody sites (34, 35, 36) and only a few typical examples can be given here. Pressman et al (37) were able to show that antibodies specific to the para-azobenzene arsonate group did not react with apparently similar molecules in which the arsonate group had been replaced by a carboxyl or sulphonate group, yet did show the same affinity for the phosphonic derivative. This demonstrated that the nature of the charged group played an important role in the specificity of the reaction in as much as the arsonate and phosphonate groups were approximately of the same size, while the other two groups were much smaller. In a

similar experiment, using antiserum homologous to the 4-azophthalate ion, Pressman and Pauling (38) pointed out that two factors favoured binding of haptens and antibodies in general: (i) steric configuration of the hapten, and (ii) van der Waal's forces. Thus, 4-chlorophthalic acid is bound more strongly and 4-iodo-phthalic acid less strongly than the phthalic acid itself. In the former case the van der Waal's attraction was larger than the steric hindrance due to the chlorine atom; in the latter case the larger steric hindrance of the iodine atom had overcome the van der Waal's forces, which favoured binding.

Although it had been postulated for a long time that the combining site of the antibody had a charge opposite to that of the antigenic group (38, 39), it was not until 1958 that Epstein and Singer actually proved it (40). Using antibodies to the para-azobenzene arsonate group and a divalent benzene arsonic acid hapten, they showed that the $-\overset{+}{N}H_3$ group of lysine had to be ionized for association to occur. In addition to ionic and van der Waal's forces, hydrogen bonds (34) and hydrophobic bonds (41) are also thought to play an important role in the reactions between antigenic and antibody combining sites.

IN VITRO MANIFESTATIONS OF ANTIGEN-ANTIBODY REACTIONS

The Precipitin Reaction

The interaction between antibodies and their appropriate antigens may result in different in vivo and in vitro manifestations (16, 42), the most common detectable in vitro reaction being the formation of a flocculent precipitate. In general, the amount of precipitate formed on addition of increasing amounts of antigen to a constant amount of antiserum is represented quantitatively by a typical curve, known as the precipitin curve (43, 44, 45) (Fig. 1). It is evident from this curve that the amount of precipitate, consisting of both antigen and antibody, increases at first in the antibody excess zone, reaches a maximum in the equivalence zone, where both reactants are precipitated quantitatively, and then decreases in the antigen excess zone, where precipitation is progressively inhibited. On the supposition that both antibody and antigen molecules had several combining sites, i.e. that these molecules are polyvalent, Pauling was able to explain the general features of these interactions in terms of his framework theory (20). Accordingly, in the region of antibody excess the precipitate would consist of small aggregates composed primarily of antibody molecules cross-linked by the small number of antigen molecules; in the region of maximum precipitation the antibody-antigen complexes would be cross-linked into larger and more compact aggregates consisting of an

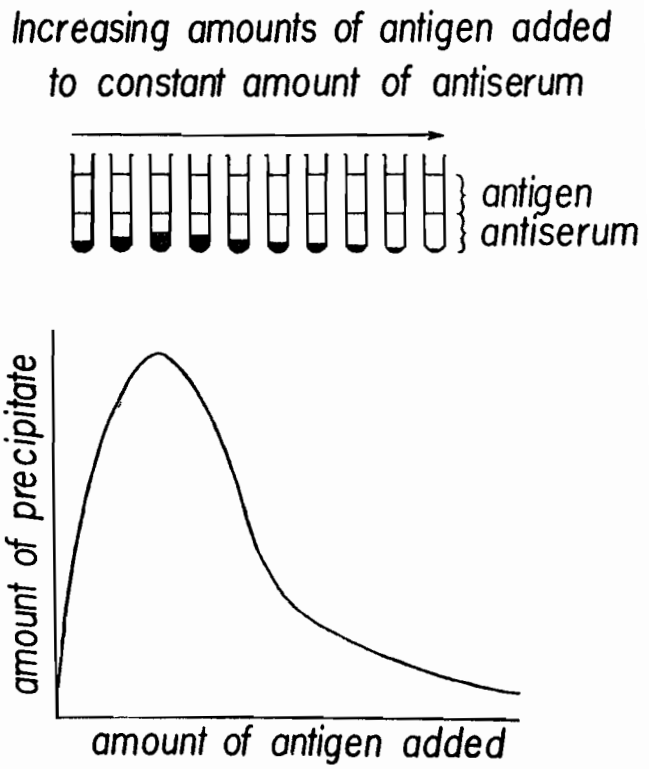

Fig. 1. Precipitin curve.

alternating and recurring antibody-antigen pattern. Addition of more antigen than that required to combine with all antibody sites would result in the disruption and loosening of this compact regular framework, and in the formation of smaller aggregates. In the limit, in excess antigen, only small complexes would be formed, consisting of one antibody molecule combined with the number of antigen molecules equivalent to the valency of the former; no cross-linking of these complexes could occur and these complexes would remain in solution. As a corollary, if the antigen, or the antibody molecule were univalent, polymeric aggregates could not be formed and the corresponding antibody-antigen complexes would be soluble.

This theory has been fully confirmed by more recent experimental data; most antigens were shown to be polyvalent. Thus ovalbumin, thyroglobulin and viviparus hemocyanin have 5, 40 and 231 antigenic sites, respectively (46). Precipitating antibodies were shown to possess two combining sites as mentioned above. On the other hand, univalent fragments I and II of rabbit antibodies prepared by enzymatic digestion, as mentioned on page 4 did not precipitate.

Hapten Inhibition Reaction

Closely related to the precipitin reaction is the hapten inhibition reaction. Landsteiner and van der Scheer (47) observed that specific precipitates consisting of hapten-protein conjugates and their homologous antibodies could be

dissolved by the addition of free hapten. Pauling et al (48) developed a theory which quantitatively described this reaction. Application of the technique and theory of hapten inhibition helped to solve a great number of problems regarding the molecular complementariness of combining sites.

Equilibrium Dialysis

Hapten-antibody reactions can be most conveniently studied by the method of equilibrium dialysis (49, 50). In this technique two compartments are separated by a semi-permeable membrane, one of the compartments contains the antibody solution, and the other a solution of the diffusible hapten. The concentration of the hapten in the latter compartment is determined prior to dialysis and after equilibrium is reached, and the amount of hapten bound is computed from the difference of the two determinations. Equilibrium dialysis has been used mainly for the determination of the thermodynamic data of antibody-hapten reactions.

Gel Diffusion Techniques

This technique was discovered in 1905 by Bechhold (51). It is based on the same principles as the precipitin reaction in as much as cross-linking between antigen and antibody occurs forming a three-dimensional network. Antigen and antibody are allowed to diffuse toward each other in a medium of agar gel. In the region where both reactants are in optimum proportions, which corresponds to the peak of the precipitin curve, a band consisting of antibody-antigen-aggregates develops. This precipitate is stabilized physi-

cally by the matrix of the agar gel.

The diffusion method was more recently improved by Ouchterlony (52) and Oudin (53). Using their refined techniques it became possible to detect multiple antibodies in a single serum. Since different antigens diffuse with different rates in agar gel medium, a number of bands will appear. This number will be equal to the minimum number of antigen-antibody systems. A quantitative application of the gel diffusion technique makes it possible to measure antigen-antibody precipitates containing as little as one microgram of nitrogen (54). A combination of double diffusion techniques with electrophoresis led to a new method called immuno-electrophoresis (55), which constitutes a powerful method for the identification of mixtures of proteins.

Agglutination Reactions

The principle of the agglutination techniques consists of the adsorption of the antigen to some particulate matter, such as collodion particles (56) or red cells pretreated with tannic acid (57) and in the clumping of these "sensitized" particles by the homologous antibodies. Fundamentally, the mechanism of the agglutination reaction has been shown to be similar to that of the precipitin test in as much as the antigen molecules are linked together by divalent antibodies (58). Since in the agglutination tests the mass of the antigen is increased by the particulate matter, much less antibody is

needed to produce a visible reaction than with soluble antigens.

To prevent desorption of the antigen from red cells, a method was devised by which the antigen could be coupled to the red cells via stable covalent bonds, e.g. using bis-diazotized benzidine (59, 60, 61) or tolylene-2,4,-diisocyanate (62). With this method antibodies can be detected in concentrations as low as 0.001 µg/ml (63).

CHAPTER II

THE PURIFICATION OF ANTIBODIES

Since the discovery of antibodies and their role in immunity, many attempts have been made to isolate antibodies in a pure form. The large number of studies devoted to the preparation of "pure" antibodies can be divided into two groups according to the principles underlying the experimental methods used: (i) non-specific methods, and (ii) specific methods. The non-specific methods of purification involve the fractionation of antisera on the basis of their physico-chemical properties, which are usually common to both antibody and normal globulins and, therefore, lead at best only to fractions enriched in their antibody content. On the other hand, in the specific methods the antibody is isolated from specific antibody-antigen complexes, thus taking advantage of the specificity of antibody-antigen interactions.

Non-specific methods

In these methods the precipitation of globulin fractions containing antibodies is achieved by the use of salts (ammonium sulphate, sodium sulphate) or of organic solvents (methanol, ethanol, acetone, ether), which lead to the dehydration of serum proteins and to a decrease in their solubility. In the majority of the fractionation methods with salts, only

heterogeneous mixtures of serum proteins can be obtained. Using a more refined fractional precipitation procedure with phosphate solutions of different ionic strength at pH 6.5, Derrien (64) succeeded in isolating at least seven different globulin fractions. More exhaustive studies were carried out on serum fractions prepared from immune sera by isoelectric precipitation at low ionic strength. In the case of horse antisera it was observed that antitoxins were confined to the pseudoglobulins (65), and anti-pneumococcus antibodies were concentrated in the euglobulins (66, 67).

Fractionation of sera with cold ethanol was developed by Cohn et al (68) and was widely used for large-scale preparation of human gamma-globulins for therapeutic or prophylactic purposes. The use of organic solvents is preferred to that of salts because it eliminates dialysis, it can be done under more sterile conditions, and gives more homogeneous preparations. The solvent most commonly employed is ethanol, because when used under strictly controlled experimental conditions, such as pH, ionic strength, temperature, protein and ethanol concentration it leads to practically complete recovery of the antibody activity (69, 70, 71, 72). The ratio of antibodies to normal proteins could thus be increased by a factor of five to seven.

In other methods antibody-enriched protein fractions are procured using certain cations or anions capable of forming protein-ion complexes with distinct solubility properties. Thus,

Isliker and Antoniades (73) succeeded in purifying antibodies by a factor of two by extracting the gamma-globulin fraction precipitated in the presence of 5 mM zinc lactate with different concentrations of glycine and tartrate. On the other hand, addition of aluminum chloride at pH 4.7 to human sera resulted in precipitation of all serum proteins with the exception of gamma globulins. This method led to a recovery of 80 to 90 percent of the gamma globulins, having a purity of over 95 percent (74). Even higher yields and purer fractions were obtained for the preparation of gamma globulins by using rivanol(2-ethoxy-6,9 -diamino-acridine-lactate) (75). Similarly, certain negatively charged polymers, such as polyacrylate (76), polymethacrylate (77) and polystyrene sulphonates (78) have been employed for gamma globulin precipitation. In one instance, the diphtheria antitoxin content of the purified fraction exhibited a fortyfold increase with respect to the antibody content of the original serum (76). Using potassium acid phthalate at pH 3.6 Northrop and Goebel (79) were able to precipitate all the normal gamma globulin, leaving a globulin solution which was 98 percent precipitable with pneumococcus polysaccharide. This, however, was an exceptional result.

In general it may be said that the non-specific methods of antibody purification led to good yields but low purities in terms of antibodies.

Specific Methods

In principle, antibodies could be isolated in a pure form by exploiting their property of combining specifically with their homologous antigens. Such a procedure would involve the following steps: (i) formation of insoluble antibody-antigen complexes, (ii) removal by washing of all other serum proteins, (iii) dissociation of these complexes, and (iv) separation of the antibodies from the antigens. The greatest difficulty encountered in this procedure is the dissociation of the antibody-antigen complexes. These specific methods can again be divided into two groups, based on the method of dissociation: (i) dissociation by non-specific means: e.g. changing the pH, the temperature or salt concentration, and (ii) dissociation by specific means, applicable only to the case of antihapten antibodies, by using an excess of hapten to dissociate antibody-antigen precipitates.

Since some antibody-antigen reactions were shown to be exothermic (80) it would be expected that raising the temperature would cause at least partial dissociation of the antibody-antigen complexes to occur. In fact, Pressman et al (81) demonstrated that antibodies to liver, kidney, lung and spleen which had been absorbed onto the cellular fractions of these organs could be eluted at 60°C without impairing their combining capacity with the tissue antigens.

In systems where the antigen is a polysaccharide,

antibodies can be liberated from the antibody-antigen complexes with concentrated salt solutions. Using 15 percent NaCl solutions, Heidelberger and co-workers (82, 83) succeeded in eluting antibodies from specific precipitates consisting of pneumococcus polysaccharides and the homologous antibodies produced in different species. This method gave yields of 0.5 to 24 percent with respect to the antibody present in the original antiserum and the purity of the antibody preparations was 60-100 percent. In spite of the fact that salt dissociation yielded antibodies of high purities, it has not been used very often because of low yields. Moreover, it does not seem to be applicable for the recovery of antibodies to proteins.

It has become increasingly more evident that antigen-antibody reactions are, at least in part, the result of electrostatic interactions between the combining sites of the antigen and antibody molecules. Therefore, one would expect that dissociation of antibody-antigen complexes would occur by modifying the electric charge of the groups involved in the antibody-antigen bonds. Thus, several investigators (84, 85) have shown that between pH 4.5 and 11 no antibody was released from complexes, while at the extreme ranges of the pH scale all complexes were dissociated.

The first attempts to elute antibodies with acid were made by Ramon (86) who recovered fairly pure diphtheria antitoxin in a yield of 75 percent from toxin-antitoxin floccules,

with 0.01 N acetic acid at 60°C. Similar results were obtained by Liu and Wu (87) and by Lee and Wu (88) in pneumococcus antibody-antigen systems. Campbell and Lanni (15) showed that anti-ovalbumin antibodies were removed from rabbit antisera by ovalbumin rendered insoluble by heat-coagulation or by surface-denaturation. Antibodies in good yields and high purity could be recovered by acid dissociation at pH 3.2. In order to purify antibodies to proteins Sternberger and Pressman (89), reacted the antigen with diazotized arsanilic acid, and subsequently the conjugates were used to precipitate the antibodies. After dissociation of the specific precipitate with $Ca(OH)_2$, the modified antigen could be removed from the solution with calcium aluminate, at pH 12. By this method yields of the order of 30 percent could be achieved. The same principle was applied by Haurowitz et al (90), the only difference being that dissociation was effected by acid in the presence of 5 percent NaCl; the azoprotein antigen was insoluble under these conditions. Singer et al (91) developed a general method for the specific purification of antibodies. They precipitated the antibodies with protein antigens to which N-acetylhomocysteine thiolactone had been coupled. The specific precipitate was dissociated in glycine-H_2SO_4 buffer of pH 2.4, and the thiolated antigen was removed by precipitation with the bifunctional organic mercurial 3,6-bis(acetoxymercurimethyl)-dioxane. Yields ranged from 30-78 percent and the purity of

the preparations was reported to be of the order of 90 percent.

The common feature of the specific purification methods discussed above is that the isolation of antibodies of high purity was accomplished only in systems in which the antigen was insoluble or was rendered insoluble during the procedure used for dissociation. In such systems the antibody, after dissociation from antigen, was readily separated from the latter by centrifugation. Obviously, these specific methods could be generally applied for the purification of antibodies if the antigens were attached to some insoluble supporting media. This principle has been employed by several investigators using different insoluble immunosorbents.

Antigens were absorbed to kaolin (92), charcoal (93) or glass beads (94) and these antigen-coated particles were shown to remove specifically antibodies from the homologous antisera. The adsorption of the antigens was sufficiently strong to allow the elution of antibodies without dissociating the antigens from the adsorbents. Yet the possibility that some antigens may become desorbed during dissociation cannot be ruled out.

Better results might be expected if the antigen were coupled to the supporting medium by stable covalent bonds. Landsteiner and van der Scheer (47) were the first to use specific immunosorbents prepared by coupling diazotized haptens to red blood cell stroma. With these conjugates anti-hapten antibodies were removed from homologous antisera and dissociation

was brought about using dilute acetic acid. This procedure led to antibody preparations which were only 50 percent pure, as determined by the precipitation of the recovered antibodies with appropriate protein-hapten conjugates. A similar method was employed by Eisen and Karush (95), who obtained preparations with a purity of 90 percent. Campbell et al (96) developed a more general method for the purification of antibodies by coupling protein antigens to a cellulose derivative. Antibody solutions in good yields (about 90 percent) and of high purity (of the order of 90 percent) were obtained. However, other investigators using the same technique were not able to show such encouraging results (97). Recently Gourvich et al (98) prepared an immunosorbent using cellulose as the supporting medium and reported that the capacity of their immunosorbent was 330 mg of antibody per 1 g of sorbent.

Ion-exchange resins have also been used as starting materials for the preparation of insoluble immunosorbents. Isliker (99) converted carboxylated or sulphonated resins to the corresponding acyl or sulphonic chlorides which were then treated with protein antigens. However, the recovery of antibodies was poor.

In the belief that supporting media with hydrophilic groups would possess properties resembling those of ion-exchange resins and would thus conceivably bind proteins also non-specifically, Gyenes and Sehon coupled protein antigens through azo bonds to a non-polar polystyrene framework (100).

Whilst this method was found to yield antibody preparations, which were 80 percent pure, the recoveries of antibodies were seldom higher than 35 percent. Similar results were obtained by other workers (101).

All the methods for the isolation of antibodies in a pure form, described so far have one common feature. They are based on a non-specific dissociation of the antibodies from the antigens by acid treatment, which may result in some denaturation of the antibodies (161). A more gentle method of dissociation can be employed for purification of anti-hapten antibodies, using an excess of hapten. Thus, Campbell et al (102) used a polyhaptenic azo dye, which was a trisubstituted derivative of resorcinol, for the precipitation of the homologous antibodies. The antibodies were dissociated from the dye by addition of an excess of the free hapten. The dye was precipitated at pH 3.5 and any dissolved free hapten was removed by dialysis. By this procedure antibodies were obtained in a yield of 90 percent and a purity of 90 percent. However, Epstein et al (103), using the same procedure, reported yields of only 45 percent. Karush and Marks (104) developed an alternate method by coupling haptens to fibrinogen, a protein which is less soluble than the antibody globulins. The antibodies to haptens were first precipitated by the addition of fibrinogen-hapten conjugates and subsequently dissociated off with a solution of free hapten. The fibrinogen-hapten conjugates were rendered insoluble in the presence of hapten and 0.05M phosphate. The

yield of antibodies was 50 percent but the degree of purity of the antibody preparation was not indicated. Another method using hapten dissociation was devised by Farah et al (105). In order to purify antibodies specific to the 2,4-dinitrophenyl group (DNP), antibodies were precipitated with a DNP-BGG conjugate. The antibody-antigen complexes were dispersed in phosphate-saline solution containing also 2,4-dinitrophenol and streptomycin. Since the streptomycin precipitated the highly anionic DNP-BGG conjugates (the conjugate was rendered highly anionic by the reaction to the ϵ-NH_2 groups of BGG with the hapten), the pure antibody remained in the supernatant. Yields of 40 percent and a purity of 90 percent was reported for these preparations.

From the results discussed in this Chapter it can be concluded that for the isolation of antibodies in a pure form specific purification methods are to be preferred to non-specific methods, which cannot distinguish between immune and normal gamma-globulins. Moreover, it would appear that dissociation of antibody-antigen complexes by acid may lead to some structural modification of the antibodies, and that milder conditions can be maintained if dissociation can be achieved at neutral pH with an excess of hapten. Unfortunately, the latter method can only be used only for antibody-hapten systems. If the antigenically determinant groups of complex antigens were known, this method could also be extended to antibody-antigen systems by using as eluents low molecular weight compounds with structures identical to those of the determinant groups.

CHAPTER III

PHYSICO-CHEMICAL STUDIES OF ANTIBODY-ANTIGEN AND ANTIBODY-HAPTEN INTERACTIONS

With the realization that antisera produced against an antigenically determinant group could contain a spectrum of antibodies directed against different portions of the determinant, Pauling et al (106) postulated the concept of heterogeneity of antibodies with respect to their binding affinity for the antigenic group. He showed that the distribution of the association constants for these systems could be described by a Gaussian error function. In consequence, similar considerations also hold for the distribution of the corresponding free energies.

This concept, in conjunction with the technique of hapten inhibition, was used with great success to investigate the molecular parameters governing antigen-antibody reactions (34). It was shown that in addition to steric factors determining the complementariness of antigen-antibody sites, their binding was due to the interaction of opposite charges and of van der Waals' forces (34). More recently, the participation of hydrogen and hydrophobic bonds in these reactions has been also invoked (41). Using the hapten inhibition method, Nisonoff et al (35, 36) were able to determine the contribution of the different parts of a hapten molecule to the overall free energy of binding. Thus, antibodies to 3-azopyridine showed

distinct specificity for the ring nitrogen atom (35), and the contribution of the latter to the free energy of binding was estimated to be -2.4 kcal/mole greater than that of a C-H group in the same position. Similarly, the nitro group was shown to contribute -5.3 kcal/mole in the association reaction between 3-azonitrobenzene and the homologous antibodies (36).

Another important contribution to the understanding of antibody-antigen reaction was made by Goldberg (107). The choice of particular distribution functions to describe appropriately experimental systems is essential for the development of a theory of antibody-antigen reactions. Goldberg (107) chose the most probable distribution, i.e. one which can be formed in the greatest number of ways. Since the most probable distribution is the one which corresponds to maximum entropy, Goldberg's choice favours the most probable path, i.e. the path of maximum entropy, to equilibrium. In order to permit the mathematical operation necessary for the development of his theory, Goldberg found it necessary to make two assumptions: (i) the non-existence of cyclical structures and (ii) the equivalence of all antigen and all antibody sites regardless of the size or shape of the complex in which they may be incorporated. On the basis of these considerations, Goldberg was able to predict the distribution of antibody-antigen complexes for any antibody-antigen ratio.

With the aid of the Goldberg theory it became possible to establish binding constants and related thermodynamic para-

meters for antibody-antigen reactions using a combination of ultracentrifugal and electrophoretic techniques (21, 108, 109). To avoid the formation of precipitates, the reactions were investigated using soluble antibody-antigen complexes in the antigen excess zone. These studies yielded important thermodynamic data (see Table I) and have also provided direct evidence for the correctness of the essential features of Pauling's framework theory. Moreover, from the pH dependence of the binding constants, it was concluded that a single ionized carboxyl group is involved in the binding of bovine serum albumin and ovalbumin with their appropriate antibodies (110, 111).

The reaction between a divalent hapten, terephthalanilide-p,p'-diarsonic acid, and antibodies directed against benzene arsonic acid (103), as well as the reaction of these antibodies and a univalent antigen, consisting of a derivative of mercaptalbumin with N-(p-benzene arsonic acid)iodoacetamide (112), was investigated by light scattering. In the former reaction linear aggregates were produced.

The most direct and least equivocal method available for the determination of the equilibrium constants of reactions between univalent haptens and the homologous antibodies is equilibrium dialysis. The experimental data can be treated by several methods, all of which are derived from the law of mass action. A detailed description of the various mathematical relationships has been given by Klotz (113). The basic assumption made

in these derivations was that the protein molecule has n distinguishable sites each of which has the same intrinsic affinity for the binding of small molecules. The fundamental relationship is represented by the equation

$$\frac{1}{r} = \frac{1}{nK}\frac{1}{(c)} + \frac{1}{n}$$

where r is equal to the moles of small molecules bound per mole of protein, c is the concentration of the unbound small molecules, and K is the intrinsic equilibrium constant. Furthermore, it was shown (113) that the experimental equilibrium constant, K_i, for the formation of the ith complex is related to the intrinsic association constant K by the equation

$$K_i = \frac{n-i+1}{1} K$$

Karush and Sonenberg (114) used a more realistic approach, as also suggested by Pauling et al (106), by including in their treatment the possibility of the n sites having independent binding constants. Thus, the normalized Gaussian error function for the free energies of binding is represented by the distribution function

$$\frac{1}{\sqrt{\pi}\sigma} \exp\left(-\frac{1}{\sigma} \ln \frac{K}{K_o}\right)^2$$

where σ is a measure of the range of values of K, and K_o is the average binding constant. Considering an infinitesimally small region, the fraction of total sites, n, on the molecule with a specific binding constant, K, may be expressed as

$$\frac{dn}{n} = \frac{1}{\sqrt{\pi}\sigma} \exp\left(-\frac{1}{\sigma} \ln \frac{K}{K_o}\right)^2 d\left(\ln \frac{K}{K_o}\right)$$

With the aid of this equation, assuming that the $\underline{n}$ sites act independently, and denoting the concentration of unbound molecules as $\underline{c}$, it was shown (113) that

$$\frac{r}{n} = 1 - \frac{1}{1 - f(c)}$$

where $$f(c) = \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} \frac{\exp(-\alpha^2)}{1 + K_o c \exp(\alpha\sigma)} d\alpha$$

and $$\alpha = \frac{\ln\left(\frac{K}{K_o}\right)}{\sigma}$$

Thus, it can be deduced that $f(c) = 1/2$ for all σ values when $K_o c = 1$.

Theoretical curves of $\frac{n}{r}$ versus $\frac{1}{K_o(c)}$ have been calculated for a variety of σ. The value of σ which best describes the experimental data obtained, is taken as the heterogeneity index for the particular system under study.

A similar approach was used by Nisonoff and Pressman (115) for antibody-hapten interactions with the aid of the equation

$$\frac{1}{F_B} = \frac{1}{(K_o c)^a} + 1$$

which has been originally derived by Sips (116) for the adsorption of a gas on a solid surface. In this relation F_B represents the fraction of antibody sites occupied by hapten molecules, K_o the average equilibrium constant, $\underline{a}$ the heterogeneity index

and $\underline{c}$ corresponds to the free hapten concentration. Thus, $K_o = \frac{1}{c}$, when half the antibody sites are occupied. The above equation predicts that a linear relationship exists between $\frac{1}{b}$ and $\frac{1}{c^a}$, where $\underline{b}$ is the concentration of the bound hapten. Therefore, the value of $\underline{a}$ may be obtained by plotting experimental values of $\frac{1}{b}$ versus $\frac{1}{c^a}$ for various values of $\underline{a}$. The value of $\underline{a}$ which gives the best straight line represents the heterogeneity index for the system studied. When $\underline{a} = 1$, all the antibody sites have the same affinity for the hapten, which means that the system studied is homogeneous.

The majority of thermodynamic constants are calculated from the temperature coefficients of the respective equilibrium constants according to the following equations:

$$\Delta F^o = -RT\ln K_o,$$

$$\frac{d\ln K_o}{d\left(\frac{1}{T}\right)} = -\frac{\Delta H^o}{R},$$

$$\Delta F^o = \Delta H^o - T\Delta S^o$$

Some of the data obtained for various systems are listed in Table I.

The ΔF values for antibody-antigen and antibody-hapten reactions are approximately of the same magnitude as those for the binding of small molecules to serum albumins (41, 123). This might be at first sight somewhat surprising, since antibody-antigen reactions are highly specific, while albumin

TABLE I

Thermodynamic Constants for Antibody-Antigen Reactions

System	ΔF^{o} (kcal/mole)	ΔH^{o} (kcal/mole)	ΔS^{o} (e.u.)	Reference
Benzoic acid: anti-p-azobenzoate antibodies	-6.1			(117)
ϵ-N-dinitrophenyllysine: anti-DNP-antibodies	-6.8	-1.6	17	(118)
ϵ-N-dinitrophenyllysine: anti-DNP-antibodies	-11.3	-8.6	9	(119)
p-(p-dimethylaminobenzeneazo)-phenyl β-lactoside(Lac-dye): anti-Lac-dye antibodies	7.09	-9.7	-8.8	(120)
D-phenyl-(p-(p-dimethylaminobenzeneazo)-benzoyl-amino)-acetate (D-I_p-dye): anti D-I_p-dye antibodies	-7.25	-7.1	0.3	(121)
p-(tyrosineazo)-benzene sulphonic acid: anti-p-azobenzene sulphonate antibodies	-8.97	-8.39	2	(122)
Terephthalanilide-p,p'-diarsonic acid: anti-benzene arsonate antibodies	-7.4	0.8 $\pm$ 26	22 $\pm$ 9	(103)

TABLE I (Continued)

System	ΔF^{o} (kcal/mole)	ΔH^{o} (kcal/mole)	ΔS^{o} (e.u.)	Reference
Bovine serum albumin (BSA): anti-BSA antibodies	-5.5 ± 0.2	0 ± 2	20 ± 8	(108)
Ovalbumin: antiovalbumin antibodies	-5.6 ± 0.2	0 ± 2	20 ± 8	(21)
Multivalent BSA-azobenzene arsonic acid: anti-benzene arsonate antibodies	-4.8 ± 0.2	0 ± 1	18 ± 4	(109)

binds a great variety of organic and inorganic ions and molecules. To explain the supposedly "non-specific" binding between albumin and a variety of compounds, it has been suggested that the distinctive feature of serum albumins is their configurational adaptability (124). Thus, the protein molecule can assume a large number of equipotential configurations which are in equilibrium with one another. Some of these configurations may then be stabilized by the interaction with a particular molecule. The relatively small values of ΔF for antibody-antigen reactions indicate that, in spite of their specificity, the binding between antibody and antigen molecules is rather weak.

Another unusual feature of the results listed in Table I is the positive change in entropy and the almost negligible change in enthalpy associated with some of these reactions. These results are again similar with those obtained for the binding of albumin with small molecules such as dyes. One would expect that the aggregation of molecules would be associated with a decrease, rather than an increase, in entropy. To explain the apparent discrepancy for both antibody-antigen and albumin-dye interactions, it has been suggested that these reactions are accompanied by a release of the water of hydration from the binding sites (125); this effect thus compensates for any loss in entropy due to the associations of the reaction partners, and the net result is a positive entropy change. On the

other hand, Karush (126) offered the explanation that the increase in entropy was due to a molecular rearrangement in the albumin or antibody molecule to a less rigid configuration.

According to Kauzmann (41), the increase in entropy for these reactions can be explained by postulating that hydrophobic bonds are formed with the simultaneous release of water molecules. By contrast, Klotz (127) proposed that binding of small organic molecules to albumin causes non-polar side chains of the protein to be exposed, thus allowing water molecules to form a cage-like, crystalline arrangement around these side chains. It is difficult to see how the latter could be compatible with the ΔS values obtained for antibody-antigen and albumin-dye reactions.

It is interesting to note that the highest positive ΔS values were obtained for protein antigen-antibody systems (108, 21, 109). Antibody-hapten reactions in general seem to show a smaller increase in entropy and a larger negative ΔH value, but even with these simpler systems one finds some inconsistencies. Thus, for example, with reference to the results listed in Table I under (118) and (119), different thermodynamic constants were obtained, using two different antibody preparations, specific to the same hapten molecule. The data given under reference (122) represent another extreme example where a negative ΔS value was observed. The large differences in these thermodynamic constants may be accounted for in terms of the concept of heterogeneity of antibodies and in terms of the different binding forces which may govern

various antibody-hapten or antibody-antigen interactions. In addition, the degree of heterogeneity varies with the antibody preparations used. Thus, on the basis of the available data it is not possible to explain all antibody-antigen reactions in terms of a single unified mechanism.

Although it has become a general practice to utilize the methods of chemical kinetics for the elucidation of the mechanisms of most chemical reactions, only very few studies of the speed of antibody-antigen associations have been made. In some of these studies, for systems containing polyvalent antigens and divalent antibodies, kinetic data were obtained by light scattering, from measurements of the intensity changes in the light scattered as a function of time (128, 129, 130). It is conceivable, therefore, that the results obtained with such systems represent complex overall reactions, leading to the formation of aggregates of different size, and that the initial phases of these reactions were obscured by re-equilibrium steps resulting in some statistical distribution of these aggregates with respect to their size (131). In other instances, such as the kinetic studies of the inhibition of luciferase (132) and of the neutralization of bacteriophage by the corresponding antibodies (133), rates for the combination of antigen with antibody were not measured directly. Berson and Yalow (134) studied the kinetics of the interation of insulin with its specific antibodies. Using I^{131}-labelled insulin they were

able to employ concentrations of reactants as low as 1×10^{-9} M. The forward rate constants obtained by these authors ranged between 1×10^{4} M^{-1} sec^{-1} and 8×10^{5} M^{-1} sec^{-1}.

Recently an attempt was made in this laboratory (135, 136) to investigate the kinetics of antibody-hapten reactions, with the aid of cathode-ray polarography. The system which was studied consisted of the reducible hapten, 4-(4'-aminobenzeneazo)-benzene arsonate ion, and its homologous antibodies. The results of this study showed that the reaction had reached completion within 1 to 2 seconds, which was the time required for mixing the reactants and for starting concentration measurements. In consequence, only the lower limiting rate constants for this reaction could be calculated. Thus, for the reaction

$$Ab + H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AbH$$

the values of 1×10^{6} M^{-1} sec^{-1} and 1 sec^{-1} were derived for k_1 and k_{-1}, respectively.

A similar value for k_1 was computed by Sturtevant et al (137) from results of stopped flow experiments with a system consisting of the coloured hapten 2(2,4-dinitrophenylazo)-1-naphthol-3, 6-disulphonic acid and the antibodies to the 2,4-dinitrophenyl determinant. They found that 57 percent of the reaction was completed within the deadtime of their instrument (0.004 seconds), and derived a value of 1×10^{6} M^{-1} sec^{-1} for the slower portion of the reaction.

From the results of the last two studies it is obvious that antibody-hapten reactions are too fast to be studied by conventional techniques of chemical kinetics. Therefore, new methods, recently evolved to follow the rates of very rapid reactions (138), must be used to measure the rates of antibody-hapten reactions.

CHAPTER IV

GENERAL PRINCIPLES OF THE TEMPERATURE-JUMP RELAXATION METHOD

In contrast to standard kinetic techniques, the relaxation methods developed within the last few years lend themselves to the determination of the rates of very rapid reactions, including those of diffusion controlled reactions (138). These techniques have a wide applicability in chemical kinetics and are primarily useful in the study of the kinetics of such reactions, in which the equilibrium is reached within the time of mixing of the reagents. The equilibrium conditions of such reactions are perturbed within an extremely short time, of the order of microseconds, and the readjustment of the system to its new state of equilibrium is followed as a function of time. Perturbation of the equilibrium can be achieved by a sudden change in physical parameters such as temperature (139, 140), pressure (141) and electric field strengths (142), which relate the observed relaxation times to the rate constants of the reactions involved. As will be shown later, the results obtained by these methods may be described in terms of relatively simple mathematical expressions (138).

The first theoretical treatment of relaxation phenomena was given by Einstein (143), who considered a dissociating gas which was subjected to rapid adiabatic expansions and dilata-

tions in a sound wave. Molecular relaxation processes, resulting from energy transfer amongst different degrees of freedom were discovered by a number of investigators (144, 145, 146). A complete treatment of relaxation phenomena on the basis of the thermodynamics of irreversible processes was given by Meixner (147, 148).

As long as deviations from equilibrium are small, a linearization of the rate equation with respect to time-dependent concentration variables is permissible. Thus, in general, the rate of disappearance of the small difference between the concentration at any time and the equilibrium concentration is proportional to this difference, i.e. $-\frac{d\,\Delta c'}{dt} = \frac{1}{\tau}\,\Delta c'$. The reciprocal of this proportionality factor has the dimensions of time and is called the relaxation time.

For a one-step reaction of arbitrary order the linearized rate equation can be expressed as

$$\frac{d\Delta c}{dt} = \frac{1}{\tau}\left[(\bar{c}-c^{o}) - (c-c^{o})\right] = \frac{1}{\tau}(\Delta\bar{c}-\Delta c) \qquad (1)$$

where $\Delta\bar{c}$ is the difference between the equilibrium concentration $\bar{c}$ and a time independent reference value c^{o} and Δc is the difference between the actual concentration c and a time independent reference value c^{o}. In practice c^{o} and $\bar{c}$ represent the concentrations of one reacting species, prior to perturbation of the system and after the system reaches its new equilibrium, respectively.

Reactions which are associated with a change of enthalpy can be most readily perturbed by a sudden jump in

temperature. This can be achieved by discharging a condensor through conducting solution containing the system to be studied. The discharge of a condensor obeys the exponential law

$$V_t = V_o \exp\left(-\frac{t}{RC}\right) \tag{2}$$

and the temperature rise is given by

$$\Delta T_t = \int_o^t \frac{V_o^2 \exp\left(-\frac{2t}{RC}\right)}{C_p R}\, dt \tag{3}$$

where ΔT_t = temperature rise at time t

R = resistance of the circuit

C = capacitance of the condenser

V_o = initial voltage of the condenser

V_t = voltage of the condensor at time t

C_p = heat capacity of the solution

If C_p and R are considered as constants

$$\Delta T_t = \Delta T_\infty \left[1 - \exp\left(-\frac{2t}{RC}\right)\right] \tag{4}$$

The time constant of the temperature change is given by

$$\tau_T = \frac{RC}{2} \tag{5}$$

and the total change in temperature is given by

$$\Delta T_\infty \simeq \frac{CV_o^2}{2C_p} \simeq \frac{CV_o^2}{2c_p \rho V} \tag{6}$$

where c_p is the specific heat of the solution, ρ is the density, and V is the volume. Connected with the temperature rise there is a pressure wave which can be calculated from the equation

$$dV = \left(\frac{\partial V}{\partial T}\right)_P dt + \left(\frac{\partial V}{\partial P}\right)_T dP = 0$$

or
$$\left(\frac{\partial P}{\partial T}\right)_V = \frac{\left(\frac{\partial V}{\partial T}\right)_P}{\left(\frac{\partial V}{\partial P}\right)_T} = -\frac{\alpha}{\beta} \qquad (7)$$

where α is the coefficient of thermal expansion and β the compressibility. A temperature rise of 10°C creates a pressure of about 50 atmospheres. The equilibrium is affected for a short time of 10-15 μsec according to the equation

$$\left(\frac{\partial \ln K}{\partial P}\right)_T = -\frac{\Delta V}{RT} \qquad (8)$$

where ΔV is the difference between the partial molar volumes of products and reactants. This effect, however, may be neglected for systems, for which the relaxation time due to the temperature jump is longer than the time required for the pressure wave to leave the system.

The temperature dependance of a chemical reaction is governed by the Van't Hoff equation

$$\frac{d \ln K}{dT} = \frac{\Delta H}{RT^2} \qquad (9)$$

and for small changes

$$\frac{\Delta K}{K} = \frac{\Delta H}{RT} \frac{\Delta T}{T} \qquad (10)$$

The equilibrium constant for the reaction $AB \rightleftharpoons A + B$ can be expressed in terms of the degree of dissociation, α, by the equation

$$K = \frac{\alpha^2}{1-\alpha} C_0 \qquad (11)$$

and, therefore, from equations (10) and (11)

$$\frac{\Delta C_A}{C_A} = \frac{\Delta C_B}{C_B} = \frac{\Delta\alpha}{\alpha} = \frac{1-\alpha}{2-\alpha}\,\frac{\Delta H}{RT}\,\frac{\Delta T}{T} \qquad (12)$$

The change in concentration of a reacting component can be followed by various physical techniques depending on the properties of the system. Thus, absorption spectrometry (139, 149), fluorescence spectrometry (150), polarographic techniques (151) and conductivity measurements (152) have been used. The most versatile detection technique used to date has proved to be absorption spectrometry, because a great number of chemical compounds absorb in the visible region of the spectrum, or their reactions can be studied by the use of coloured indicator solutions (139, 153). It can be shown that for a given reactant under observation the change in light intensity ΔI is proportional to Δc (138).

It was pointed out above (equation (1)) that

$$\frac{d\Delta c}{dt} = \frac{\Delta\bar{c} - \Delta c}{\tau}$$

However, since $\Delta\bar{c}$ is time dependent and is also proportional to ΔT, using equation (4) one obtains

$$\Delta\bar{c}_t = \Delta c_\infty\left[1 - \exp\left(-\frac{2t}{RC}\right)\right] \qquad (13)$$

and substitution in equation (1) yields

$$\frac{d\Delta c}{dt} + \frac{\Delta c}{\tau} = \frac{1}{\tau}\Delta c_\infty\left[1 - \exp\left(-\frac{2t}{RC}\right)\right] \qquad (14)$$

The solution of the latter equation is

$$\Delta c_t = \Delta c_\infty \left[1 - \frac{1}{1 - \frac{RC}{2\tau}} \exp(-\frac{t}{\tau}) - \frac{1}{1 - \frac{2\tau}{RC}} \exp(-\frac{2t}{RC}) \right] \quad (15)$$

Two cases can be distinguished

(i) when $\tau \ll \tau_T = \frac{RC}{2}$

then equation (15) reduces to

$$\Delta c_t = \Delta c_\infty \left[1 - \exp(-\frac{2t}{RC}) \right] \quad (16)$$

This equation shows that variations in concentration obey the same time relationship as the variations in temperature, which means that the reaction is always in equilibrium, the concentration changes following the temperature changes immediately. Under these conditions the kinetics of the reaction cannot be studied.

(ii) When $\tau \gg \tau_T = \frac{RC}{2}$

equation (15) yields the expression

$$\Delta c_t = \Delta c_\infty \left[1 - \exp(-\frac{t}{\tau}) \right] \quad (17)$$

from which it follows that the change in concentration changes exponentially with time.

Since $\Delta c \propto \Delta I$

$$\Delta I_t = \Delta I_\infty \left[1 - \exp(-\frac{t}{\tau}) \right] \quad (18)$$

This equation represents the relationship upon which the direct determination of the relaxation time τ by absorption spectrometry is based.

If it is preferred to express the linearized rate equation (1) in terms of the deviations from equilibrium given by the higher temperature,

then $$\frac{d\Delta c'}{dt} = -\frac{\Delta c'}{\tau} \tag{19}$$

which upon integration yields

$$\Delta c'_t = \Delta c_\infty \exp(-\frac{t}{\tau}) \tag{20}$$

In other words, the relaxation time τ is that time after which 1/e of the reaction remains to be completed. In practice, the relaxation time for a given reaction is evaluated from the corresponding experimental curves.

The observed relaxation times for single step reactions are related to the rate constants, which characterize these reactions. Thus for a bimolecular reaction of the type

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AB$$

the rate may be expressed by the equation

$$\frac{d(A)}{dt} = k_{-1}(AB) - k_1(A)(B) \tag{21}$$

The concentration of the reactants at any time t, i.e. (A), (B) and (AB), may be expressed in terms of the corresponding equilibrium concentrations $(\bar{A})$, $(\bar{B})$ and $(\overline{AB})$, and small deviations from these values.

Thus
$$\begin{aligned} (A) &= (\bar{A}) + \Delta(A) \\ (B) &= (\bar{B}) + \Delta(B) \\ (AB) &= (\overline{AB}) + \Delta(AB) \end{aligned} \tag{22}$$

Substitution in equation (21) yields

$$\frac{d\left[(\bar{A}) + \Delta(A)\right]}{dt} = k_{-1}\left[(\overline{AB}) + \Delta(AB)\right] - k_1\left[(\bar{A}) + \Delta(A)\right]\left[(\bar{B}) + \Delta(B)\right]$$

$$= k_{-1}(\overline{AB}) + k_{-1}\Delta(AB) - k_1(\bar{A})(\bar{B}) - k_1(\bar{A})\Delta(B)$$

$$- k_1(\bar{B})\Delta(A) - k_1\Delta(A)\Delta(B) \qquad (23)$$

If the deviations from equilibrium are small, the last term of this relation tends to zero, and since $\Delta(A) = \Delta(B) = -\Delta(AB)$, and $k_{-1}(\overline{AB}) = k_1(\bar{A})(\bar{B})$ at equilibrium, one can write

$$\frac{d\Delta(A)}{dt} = -\left\{k_{-1} + k_1\left[(\bar{A}) + (\bar{B})\right]\right\}\Delta(A) \qquad (24)$$

But according to equation (19), $\frac{d\Delta(A)}{dt} = -\frac{\Delta(A)}{\tau}$

Thus, it follows that

$$\frac{1}{\tau} = k_{-1} + k_1\left[(\bar{A}) + (\bar{B})\right] \qquad (25)$$

The following relations have been derived for the single step reactions listed below (138).

$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B$	$\frac{1}{\tau} = k_{-1} + k_1$
$A + C \rightleftharpoons B + D$ (C = catalyst)	$\frac{1}{\tau} = k_{-1}(\bar{C}) + k_1(\bar{C})$
$2A \rightleftharpoons A_2$	$\frac{1}{\tau} = k_{-1} + k_1(\bar{A})$
$A + B \rightleftharpoons C$ (B = buffered)	$\frac{1}{\tau} = k_{-1} + k_1(\bar{B})$
$A + B \rightleftharpoons C + D$	$\frac{1}{\tau} = k_{-1}\left[(\bar{C}) + (\bar{D})\right] + k_1\left[(\bar{A}) + (\bar{D})\right]$
$A + B + C \rightleftharpoons D$	$\frac{1}{\tau} = k_{-1} + k_1\left[(\bar{A})(\bar{B}) + (\bar{A})(\bar{C}) + (\bar{B})(\bar{C})\right]$

The temperature-jump relaxation method can be used for rate measurements in the time range of 1 to 10^{-6} sec (138), and as will be shown in Chapter VI it is applicable to studying the kinetics of albumin-dye and antibody-hapten reactions.

THE SCOPE OF THE PRESENT STUDY

The present study is divided into two main parts

(i) The use of stroma-hapten conjugates for the purification of anti-hapten antibodies is described in Chapter V,

(ii) The application of the temperature-jump relaxation technique for the study of the kinetics of albumin-dye and antibody-hapten interactions is given in Chapter VI. The results derived from this investigation are reviewed in the section entitled General Discussion.

CHAPTER V

EVALUATION OF THE USE OF STROMA-HAPTEN CONJUGATES FOR THE PURIFICATION OF ANTIHAPTEN ANTIBODIES

INTRODUCTION

When this work was started it had been planned to study by light scattering the kinetics of antibody-antigen reactions, using antibodies specific to the phenyl arsonate group and a univalent antigen consisting of the conjugate of mercaptalbumin with N-(p-benzene arsonic acid) iodoacetamide.

For this purpose it was deemed necessary to use antibody solutions of high purity, since the presence of any high molecular weight impurities, such as normal gamma globulins, would have contributed greatly to the amount of light scattered and would have thus masked any increase in the light scattered due to the formation of the antibody-antigen complexes.

The use of red cell stroma in the preparation of a specific immunosorbent for the purification of antibodies to the phenyl arsonate group, appeared advantageous. Thus, red cell stroma could be obtained readily and the results of earlier studies (47, 95), reviewed in Chapter II, indicated that antibodies of fairly high purity could be recovered from such immunosorbents.

In contrast to the previous procedures (47, 95) it was decided to dissociate the antibodies off the immunosorbent with an excess of the free hapten rather than by acidification, since it had been shown that exposure of gamma globulins to

media more acidic than pH 4.2 resulted in some modification of their structure (161). Moreover, an independent study in this laboratory had demonstrated that dissociation of antibodies at low pH from an immunosorbent, consisting of polystyrene-protein conjugates, resulted in an apparent modification of the antibodies (100).

EXPERIMENTAL

Preparation of the Stroma-Hapten Conjugates

A procedure essentially similar to that of Landsteiner and van der Scheer (47) was used. Bovine erythrocytes were lysed by heating at 60°C for 1 hour, were then suspended in five volumes of water and shaken vigorously for 15 minutes. Enough sodium chloride was added to the suspension to make a 1 percent solution. The stroma was then separated by centrifugation. The stroma was repeatedly resuspended in saline solution and centrifuged until the supernatant obtained was colourless. For removal of lipids the stroma was resuspended in an equal volume of water to which half a volume of toluene was added. The mixture was shaken intermittently over a period of 48 hours and was then centrifuged. The toluene layer containing some of the lipids was discarded, and the stroma was freeze-dried. Subsequently it was extracted with ether at room temperature during 24 hours, and finally with ethanol in a Soxhlet extractor. The extracted stroma was dried.

For the coupling of arsanilic acid to the stroma,

0.5 millimoles of diazotized p-arsanilic acid per gram of dried stroma were used. The latter was dispersed in 100 ml of water using a "Virtis" homogenizer and a volume of 18 ml of a 1 M sodium carbonate solution was added. The suspension was placed in an ice bath and the diazotized p-arsanilic acid was added dropwise, with vigorous stirring of the mixture for one hour. The suspension was then acidified with hydrochloric acid to pH 3.0 and centrifuged. The stroma-hapten conjugates were resuspended in borate buffer, centrifuged and washed with the buffer (pH 8.0, $\Gamma/2 = 0.15$) at room temperature until the supernatant was colourless.

Preparation of Soluble Hapten-Protein Conjugates

On the average about 40 p-arsanilic acid residues were coupled per molecule of bovine gamma globulin (BGG) or per molecule of human serum albumin (HSA), using standard diazotization and coupling procedures. The resulting protein-hapten conjugates (BGG-R and HSA-R) were dialyzed exhaustively against a solution of bicarbonate. Both conjugates were finally dialyzed against borate buffer.

Preparation and Isolation of Antibody

Rabbits were immunized intravenously 3 times a week for periods of 4-6 weeks with 1 ml portions of 2 percent solution of BGG-R until sufficiently high-titered antisera were produced. The rabbits were bled 5 days after the last injection. After a rest period of 3-4 weeks the immunization procedure was repeated. The antisera were Seitz-filtered into sterile vials and stored at $4^{\circ}C$.

For the isolation of antibodies decomplemented sera were used. For removal of complement from anti - BGG-R sera, an equal volume of BGG solution, containing enough BGG to precipitate all the anti-BGG antibodies, was added. Subsequently, approximately 0.1 gm of packed, wet stroma-hapten conjugates was added to the antiserum, containing about 1 mg of antibody, and the suspension was stirred slowly at $4^{o}C$ for 4 hours. The immunosorbent-antibody complexes were packed by centrifugation at 2000 RPM for 10 minutes. They were washed with cold borate buffer.

For the dissociation of the antihapten antibodies, the immunosorbent-antibody complexes were suspended in a 5-10 percent solution of the sodium salt of p-arsanilic acid or the sodium salt of p-iodobenzene arsonic acid. The volume of dissociating hapten was equal to that of the antiserum used. The supernatants (hereafter referred to as eluates) were dialyzed against borate buffer (pH 8.0, $\Gamma/2 = 0.15$) in standard Visking tubing for 1 to 7 days. It was found necessary to boil the Visking tubing in distilled water for a few minutes to remove nitrogenous materials present in the interstices of the tubing. In an alternate method the antibodies were dissociated off the immunosorbents by adding hydrochloric acid to the suspension to a final pH of 3.2. The pH of the eluate was adjusted to 7.0 and the latter was dialyzed against borate buffer for 12-16 hours.

The antibody content of the whole sera and of the eluates was determined by the quantitative precipitin test, as recommended by Heidelberger and Kendall (43), and the nitrogen content of the precipitates was obtained by the micro-Kjeldahl procedure of McKenzie and Wallace (154).

Preparation of p-Iodobenzene Arsonic Acid

p-Iodobenzene arsonic acid was prepared according to the classical Sandmeyer reaction (155) and was recrystallized from 50 percent ethanol (156).

For the preparation of the I^{131}-labelled compound the recommendations of Sloviter (157) were used to destroy any free iodine produced in the reaction. A quantity of 0.67 g of p-aminobenzene arsonic acid was dissolved in 7.0 ml of water to which 0.36 ml of concentrated sulphuric acid was added and the solution was cooled to $4^{\circ}C$. For diazotization 0.21 g of sodium nitrite in 1.5 ml of water was added. Any excess of nitrous acid was destroyed with sulphamic acid. To replace the diazonium group by iodine, a solution containing 0.500 g of potassium iodide, 3 mc I^{131}-labelled sodium iodide and a small quantity of sodium sulfite was added to the diazonium salt. The mixture was allowed to stand in an icebath for 30 minutes, at room temperature for one hour, and was finally kept in a water bath at $70\text{-}80^{\circ}C$ for another 30 minutes. The precipitate was washed 4 times with a 5 percent solution of sodium bisulfite and twice with cold distilled water. Finally the precipitate

was recrystallized 3 times from 50 percent ethanol. The radioactivity of the samples was measured in a well type NaI(Tl) crystal scintillation counter* and the specific activities ranged from 2.5×10^6 to 3.5×10^6 counts per minute per milligram.

Hapten-Inhibition Experiments

To two equal portions of decomplemented anti-R antiserum optimal concentrations of HSA-R were added and the mixtures were incubated at $4^{\circ}C$ for 48 hours. The precipitates were washed with solutions of borate buffer to remove non-specific proteins. One of the precipitates was dissolved in an excess of sodium arsanilate. Both samples were transferred quantitatively into dialysis bags, and dialyzed for 72 to 96 hours against borate buffer. The amounts of precipitate in both bags were measured. The same experiment was repeated using p-iodobenzene arsonic acid as an inhibitor.

Free Electrophoresis and Ultracentrifugation

Electrophoretic analyses of whole and absorbed sera were done in a Spinco Tiselius apparatus. All experiments were performed in veronal buffer (pH 8.6, $\Gamma/2 = 0.1$) and the concentration of each of the electrophoretic components was calculated from photographs of the Rayleigh interference fringe

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* The author would like to thank Dr. L. Yaffe of the Department of Chemistry, McGill University, for placing the facilities of the radiochemistry laboratory at his disposal.

patterns of the ascending boundaries (158). An optical Spinco ultracentrifuge was used for the determination of the sedimentation properties of the various globulin preparations. For this purpose samples of eluates were concentrated by dialysis against a 20 percent solution of polyvinyl pyrrolidone (M.W. 30,000) dissolved in normal saline*. Before centrifugation the samples were dialyzed overnight against saline.

RESULTS

The antibody concentration of the anti-R antisera used in this study ranged between 1 to 2 mg/ml. Comparison of the electrophoretic patterns obtained with the antisera prior to and after absorption with stroma-hapten conjugates showed that the bulk of the serum proteins removed were γ-globulins and that smaller amounts, corresponding to about 30 percent of the total materials removed, were α and β globulins. On the other hand, no albumin was removed on absorption. The results of a typical experiment are illustrated in Table II.

Ultracentrifugal analysis of the eluates revealed that most of the material (90 percent) had a sedimentation coefficient of 7S (uncorrected), corresponding to that of

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* The polyvinyl pyrrolidone had been previously dialyzed exhaustively against distilled water for the removal of any low molecular weight components, and was dried by lyophilization.

TABLE II

Electrophoretic Analysis of Rabbit Anti-R Serum Before and After Exposure to Stroma-Hapten Conjugates

	Whole Anti-R Serum g percent	After Absorption With Stroma-R g percent
Albumin	3.45	3.45
α	0.94	0.91
β	0.78	0.75
γ	1.29	1.15

normal γ-globulins, and that the remainder had a sedimentation coefficient of 20S. The latter component might have represented some large molecular aggregates. No slower sedimenting components were detected, indicating that no albumin was present in the antibody preparations.

The results of absorption experiments demonstrated that complete removal of antibodies from homologous antisera was achieved and that about 0.1 g of wet, packed stroma-hapten conjugates was sufficient to combine with 1 mg of rabbit antibody. When antibodies were eluted with an excess of sodium arsanilate, the eluates were found to be colourless. This was taken as an indication that the hapten-stroma conjugates used in this study remained insoluble. The use of larger quantities of diazotized p-arsanilic acid in the coupling reaction, as originally employed by Landsteiner and van der Scheer (47), resulted in the production of large quantities of soluble stroma-hapten conjugates which could not be completely removed from the insoluble conjugates on prolonged washing with buffer. Occasionally, however, small insoluble particles remained in suspension and could not be removed on prolonged centrifugation at 2000 RPM, except after dialysis against borate buffer. This was probably caused by the high density of the solutions containing the large concentrations of sodium arsanilate used in these experiments.

Elution of anti-R antibodies from the immunosorbent

with sodium arsanilate, followed by dialysis of the eluates for removal of the hapten, demonstrated that the optimal yield of precipitable antibodies was obtained after dialysis for 72 hours. Thus, the recovery of precipitable antibodies after dialysis for 24 hours was about 30 percent and increased to 65 percent on increasing the period of dialysis to 72 hours. Dialysis for periods longer than 72 hours did not result in a further increase in the yield or in the purity of the recovered antibodies (Table III). These data were calculated on the basis of standard precipitin curves by subtracting the amount of HSA-R added from the total precipitate obtained at the optimal zone. Typical precipitin curves of the original antiserum and the eluted antibodies are shown in Fig. 2. The purity of the different antibody preparations, recovered after 72 hours of dialysis, which was computed in terms of the amount of precipitable antibodies and the total protein concentration of the eluates ranged from 55 to 65 percent. Reabsorption of the eluate obtained in one experiment with fresh stroma-R conjugates resulted in the uptake of 92 percent of the total proteins. By contrast, in a control experiment treating normal rabbit γ-globulins* in an identical manner with an equal portion of the same immunosorbent preparation did not lead to any detectable removal of proteins. In another experiment the whole purifi-

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* Obtained by sodium sulphate fractionation according to the method of Marrack et al (159).

TABLE III

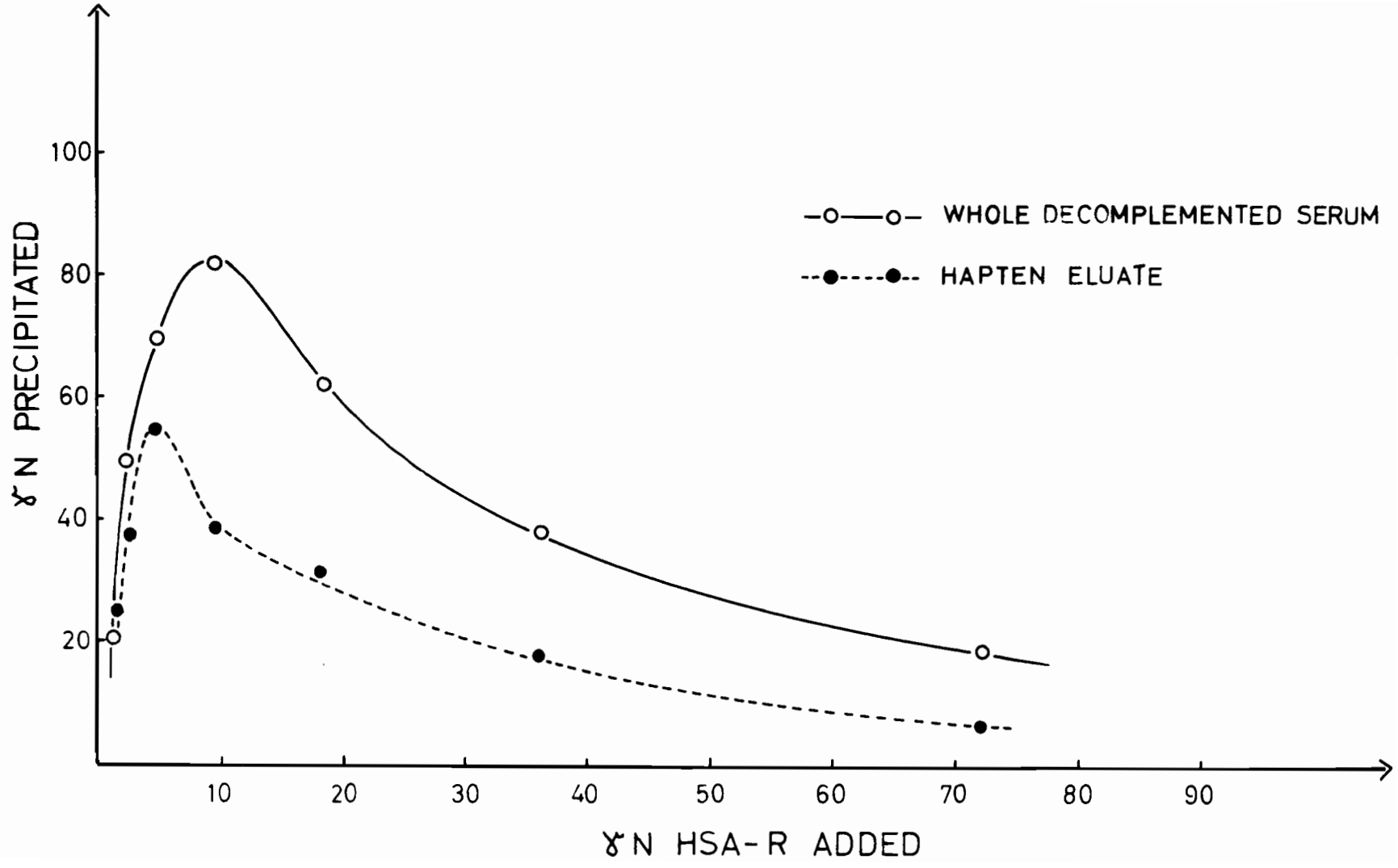
Recovery of Antibodies from Stroma-Hapten Conjugates by Hapten Elution

Expt. No.	Time of Dialysis (Hours)	Total Antibody in serum (mg/ml)	Total Protein Eluted* (mg/ml)	Precipitable Antibody in Eluate (mg/ml)	Percent Yield	Percent Purity
1	72	2.12	1.88	1.05	50	56
2	84	2.12	1.60	1.00	47	63
3	168	2.12	1.38	0.80	38	59
4	72	0.99	0.96	0.64	65	67
5	84	0.58	0.68	0.38	66	56
6	96	0.94	0.96	0.48	52	50

* These values were referred to the original volume of the serum used for absorption experiments.

FIGURE 2

Precipitin curves for whole decomplemented antiserum and for antibodies eluted from stroma-hapten conjugates


% N PRECIPITATED
100
80
60
40
20
10
20
30
40
50
60
70
80
90
% N HSA-R ADDED
WHOLE DECOMPLEMENTED SERUM
HAPTEN ELUATE

cation procedure was repeated with the eluates in an attempt to increase the purity of the final antibody preparation. This resulted in a further loss of antibodies without leading to a higher degree of purity.

The fact that the purity of the antibody preparations, as measured by the proteins precipitated with HSA-R, was lower than that calculated in terms of the material taken up by the immunosorbent on reabsorption (i.e. 65 percent as compared to 92 percent) was considered as an indication that complete precipitation of the eluted antibodies was inhibited by the eluting hapten which was strongly bound to some of the antibody molecules. To check this hypothesis, I^{131}-labelled p-iodobenzene arsonic acid* was used as the eluting hapten. The eluate was dialyzed for 72 hours. A solution containing similar amounts of normal rabbit γ-globulins and I^{131}-labelled p-iodobenzene arsonic acid served as control, and was dialyzed for the same length of time. From the difference in radioactivity of the two solutions it was calculated that about 25 percent of the combining sites of the eluted antibodies were blocked by the hapten.

Further confirmation that the low yields of precipitable antibody were attributable to inhibition by the hapten

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* This compound was used instead of an appropriately radioactive labelled p-arsanilic acid, e.g. labelled with C^{14}, because of the low yield obtained in the preparation of the latter compound and because of the prohibitive cost of the labelled starting materials.

was obtained in "hapten inhibition" experiments (as described under Methods). The results of these experiments are given in Table IV. It is evident that treatment of the antigen-antibody complexes with hapten resulted in lowering the amount of precipitable antibody in spite of extensive dialysis. This effect was more pronounced when p-iodobenzene arsonic acid was used as the inhibiting hapten, indicating that the latter hapten was more strongly bound at the antibody site than p-arsanilic acid. Independent evidence for this stronger binding of p-iodobenzene arsonic acid was also obtained when antibodies were eluted with this hapten from the immunosorbent. Thus, the purity of the antibodies eluted with p-iodobenzene arsonic acid as measured by the amount of precipitable antibodies was only about 30 percent as compared to a purity of 60 percent obtained on elution with p-arsanilic acid.

In a few experiments in which antibodies were dissociated off by lowering the pH to 3.2, the yields were of the order of 40-50 percent and the purity ranged from 60-70 percent (Table V). However, in some cases, as indicated in Fig. 3 the precipitin curves exhibited large trailing in antigen excess.

DISCUSSION

The present investigation represents an attempt to modify and improve the method of Landsteiner and van der Scheer (47) for the isolation of antihapten antibodies with stroma-hapten conjugates. As stated in Chapter II, the purity of antibody preparations obtained by these workers, on dissociation

TABLE IV

Hapten-Inhibition Experiments

		Hapten used	
		p-arsanilic acid	p-iodobenzene arsonic acid
		γ N in precipitate	
Serum A	Control precipitate after dialysis	156	156
	Precipitate treated with hapten and dialyzed	101	38
	Percent recovered	65	24
Serum B	Control precipitate after dialysis	44*	59*
	Precipitate treated with hapten and dialyzed	24	20
	Percent recovered	55	34

* These figures were obtained on two different occasions.

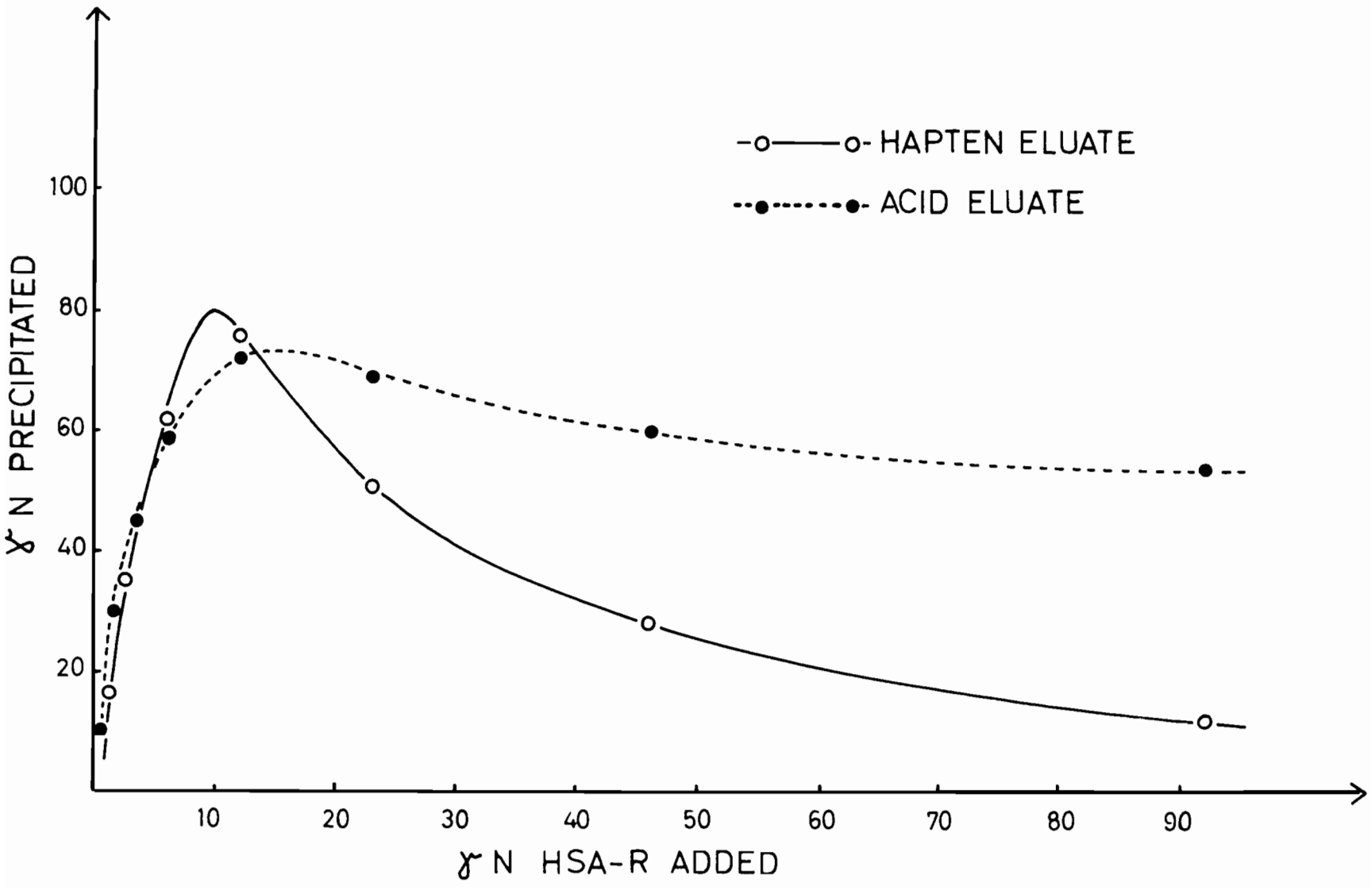
TABLE V

Recovery of Antibodies from Stroma-Hapten Conjugates by Acid Elution

Expt. No.	Total Antibody in serum (mg/ml)	Total Protein Eluted (mg/ml)	Precipitable Antibody in Eluate (mg/ml)	Percent Yield	Percent Purity
1	2.12	1.40	1.05	50	75
2	2.12	1.63	1.00	47	61
3	2.12	1.17	0.71	34	61
4	2.12	1.92	1.17	55	59

FIGURE 3

Precipitin curves for antibodies eluted with acid and hapten from stroma-hapten conjugates.


HAPTEN ELUATE
ACID ELUATE
γ N PRECIPITATED
γ N HSA-R ADDED
100
80
60
40
20
10
20
30
40
50
60
70
80
90

with acid, was of the order of 50 percent. Moreover, Eisen and Karush (95) using the same type of immunosorbent and elution procedure claimed a purity of 90 percent for their antibody preparations. However, their calculations were based on the amount of material removed from the eluates on readsorption with stroma-hapten conjugates and not on the amount of precipitable material determined directly in the eluates. Making similar assumptions, purities of the order of 92 percent could have been also claimed in this study. On the other hand, as shown in Table III, the antibody content of the eluates computed from precipitin curves amounted to 50-65 percent. The discrepancy between these data arrived at by the two different methods of calculation would indicate that some antibody sites remained blocked by the eluting hapten even after prolonged dialysis. This interpretation is further supported by the hapten-inhibition experiments, in as much as the amount of specific precipitate after dissolution with hapten and after subsequent dialysis decreased to a value of about 60 percent. These observations are in agreement with the concept of heterogeneity of antibodies (115, 160), suggesting that the hapten could be removed by dialysis only from antibody sites with low affinities, and would rule out the possibility that the eluates contained significant amounts of non-antibody proteins. The absorption experiments done with normal rabbit γ-globulins demonstrate further that there was no uptake of heterologous γ-globulins

by the immunosorbent. Similarly, no albumin was removed as shown by the electrophoresis experiments.

It is obvious that an unequivocal proof that all the eluting hapten was not removed by dialysis could have been obtained by analyzing directly the dialyzed solution for the presence of hapten. However, since the p-arsanilic acid which was inferred to have remained in the dialyzed solution would have been present only in trace amounts of the order of 10^{-8} to 10^{-9} mole/ml, no attempt was made for its quantitative analysis. Instead, I^{131}-labelled p-iodobenzene arsonic acid was used in spite of its known ability to combine more strongly than p-arsanilic acid with antibodies directed against the latter (34). As suspected, after dialysis, the eluting hapten remained attached to about 25 percent of the combining sites of the eluted antibodies. Additional evidence that some of the antibody sites were blocked by the eluting hapten was brought out by the fact that the apparent purity of the antibody preparations was lower when elution was performed with p-iodobenzene arsonic acid rather than with p-arsanilic acid.

In spite of the observation made in another study in this laboratory (100) that elution of antiprotein antibodies with acid from polystyrene-antigen complexes resulted in some structural modifications of the eluted antibodies, this method was also resorted to in the present investigation. The purity of the antibodies eluted with acid was not higher than that

obtained by elution with hapten. Moreover, from the respective precipitin curves (Fig. 3) it was evident that the antibody eluted with acid exhibited a higher degree of heterogeneity.

In conclusion, the weight of evidence obtained in the present investigation would suggest that the proteins eluted with hapten from the stroma-hapten conjugates are primarily antibody γ-globulins and that the apparent lack of purity of the antibody preparations was due to the decrease in precipitability of these antibodies caused by hapten molecules tenaciously bound to some antibody sites. While this study was in progress similar results were obtained by Farah et al (105) who demonstrated that after elution of antibodies specific to the 2,4-dinitrophenyl group from specific precipitates with various dinitrophenyl haptens, not all the eluting haptens could be dialyzed out. Attempts to completely remove these haptens by ion-exchange chromatography proved also unsuccessful (105).

CHAPTER VI

A STUDY OF THE KINETICS OF ALBUMIN-DYE AND ANTIBODY-HAPTEN REACTIONS BY THE USE OF THE TEMPERATURE-JUMP TECHNIQUE

INTRODUCTION

As mentioned in Chapter III, thermodynamic studies have yielded important information concerning the nature of the forces which operate between antibody and antigen molecules. Such studies do not provide the necessary information for the formulation of the detailed mechanism of antibody-antigen reactions. Although in general, the methods of chemical kinetics are used for the elucidation of the mechanisms of most reactions, only few kinetic studies of simple antibody-antigen reactions had been made in the past. Thus, light scattering techniques had been used to study the reactions of some protein antigen-antibody reactions (128, 129, 130). However, since in these investigations the rates were determined from the growth of the size of the antibody-antigen aggregates, it is conceivable that the initial reactions were obscured by re-equilibration steps leading to some statistical distribution of the aggregates with respect to their size.

To avoid these complications, it was decided to study the kinetics of the reactions between antibodies specific to the phenyl arsonate group and a univalent antigen consisting of a conjugate of mercaptalbumin with N-(p-benzene arsonic acid) iodoacetamide. However, during the course of this study it

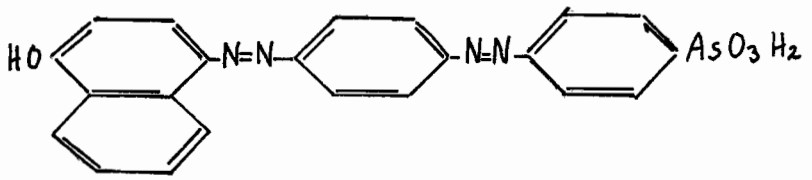
became increasingly evident from a parallel investigation that the combination of antibodies with their antigenic determinants occurred at very fast rate (24, 136), and that the technique of light scattering as well as any of the other classical methods could not be profitably applied to the kinetic studies of antibody-antigen reactions (c.f. p. 34). Therefore, the temperature-jump method, which was described in Chapter IV, was resorted to. In view of the scarcity of specific antibodies the applicability of this relaxation method to the kinetics of the reactionsof proteins with small molecules was established with a model system consisting of bovine serum albumin (BSA) and the two coloured hapten molecules 4[4-(4'-azobenzeneazo) benzene arsonic acid]-1-naphthol (N-R') and 4[4-(4'-azobenzene-azo)-benzene arsonic acid]-1-naphthol-2-sulphonic acid (NS-R'). This system was chosen since the thermodynamic constants obtained from equilibrium studies of BSA-dye interactions are similar to those of antibody-hapten reactions (41, 125).

EXPERIMENTAL

MATERIALS

Preparations of the Dye-haptens

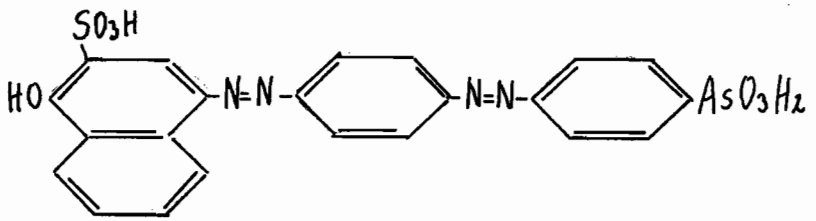
(i) The preparation of 4[4-(4'-azobenzeneazo)benzene arsonic acid]-1-naphthol (N-R')



In principle the method of preparation of this compound was similar to the one used for the preparation of Orange I (162). To 5.8 g of 1-naphthol in 250 ml of water enough concentrated NaOH solution was added to bring the pH to 10. The mixture was cooled to 0°C. To 3.1 gm of 4-(4'-aminobenzene azo) benzene arsonic acid in 25 ml of water, an equivalent amount of NaOH was added and the mixture was heated in order to dissolve the acid. After cooling, 0.75 g of $NaNO_2$ was added. The resultant mixture was poured slowly into 20 ml of 2N HCl, containing a few pieces of ice. Diazotization was allowed to proceed for 30 minutes, and the excess nitrous acid was destroyed with sulphamic acid. The diazonium compound was added slowly and with stirring to the naphthol solution. The reaction mixture was kept at pH 10 and 4°C for about one hour. The resultant compound was precipitated with hydrochloric acid and centrifuged at 4°C. It was then washed with cold water to remove any unreacted diazonium

compound. After several washings the precipitate was dissolved in NH_4OH. The azo compound was then precipitated with a solution of $MgCl_2$ and recovered by centrifugation. It was then dissolved by addition of a NaOH solution and reprecipitated by acidification with hydrochloric acid. This procedure was repeated four times. Finally, the precipitated compound was washed several times with water and dried by lyophilization.

(ii) The preparation of 4[4-(4'-azobenzeneazo)-benzene arsonic acid]-1-naphthol-2-sulphonic acid (NS-R')



This dye was prepared by a procedure similar to the one just described, using 5.2 g of the potassium salt of 1-naphthol-2-sulphonic acid and 3.1 g of 4-(4'-aminobenzeneazo) benzene arsonic acid. The sodium salt of this dye was prepared by adding a sufficient amount of sodium hydroxide solution so as to neutralize the sulphonic acid and one of the protons of the arsonate group. This salt was precipitated by adding 6 volumes of acetone and was used in all subsequent experiments.

Properties of the dye

Solutions of known concentration of the two dye-haptens were titrated with a standard solution of NaOH for the determination of the purity of the dye-haptens. From the titration curves molecular weights of 487 and 625 were calculated for N-R' and

the disodium salt of NS-R', respectively. These values were only slightly higher than the theoretical values of 476 and 600 for the corresponding compounds, and it was concluded that only negligible amounts of by-products, if any at all, had been obtained in the reactions used for the preparation of N-R' and NS-R'.

Absorption spectra of 1 x 10^{-5} molar solutions of N-R' and NS-R' were determined at various hydrogen ion concentrations, and the results are shown in Figs. 4 and 5, respectively. The absorption maxima for both dyes were shifted to higher wavelengths as the pH of the solutions was increased. This spectral shift was most likely due to the ionization of the naphtholic OH groups and to the subsequent rearrangement of the molecules to quinonoid structures. Thus, on complete ionization the absorption maxima of N-R' and NS-R' were shifted from 480 mμ to 580 mμ, and from 530 mμ to 580 mμ, respectively. The pK values of the corresponding naphtholic OH groups were determined as 8.7 and 7.2 from the appropriate optical density measurements at 580 mμ at various pH values (Figs. 6 and 7). These titration curves were obtained with solutions containing constant amounts of the dyes in the presence of buffers of different pH and at a constant ionic strength of 0.1. It should be pointed out that whilst solutions of NS-R' obeyed Beer's law over the whole concentration region used, solutions of N-R' exhibited deviations at concentrations higher than 1 x 10^{-5} M.

FIGURE 4

The effect of pH on the absorption spectrum of a 1 x 10^{-5}M solution of N-R'.

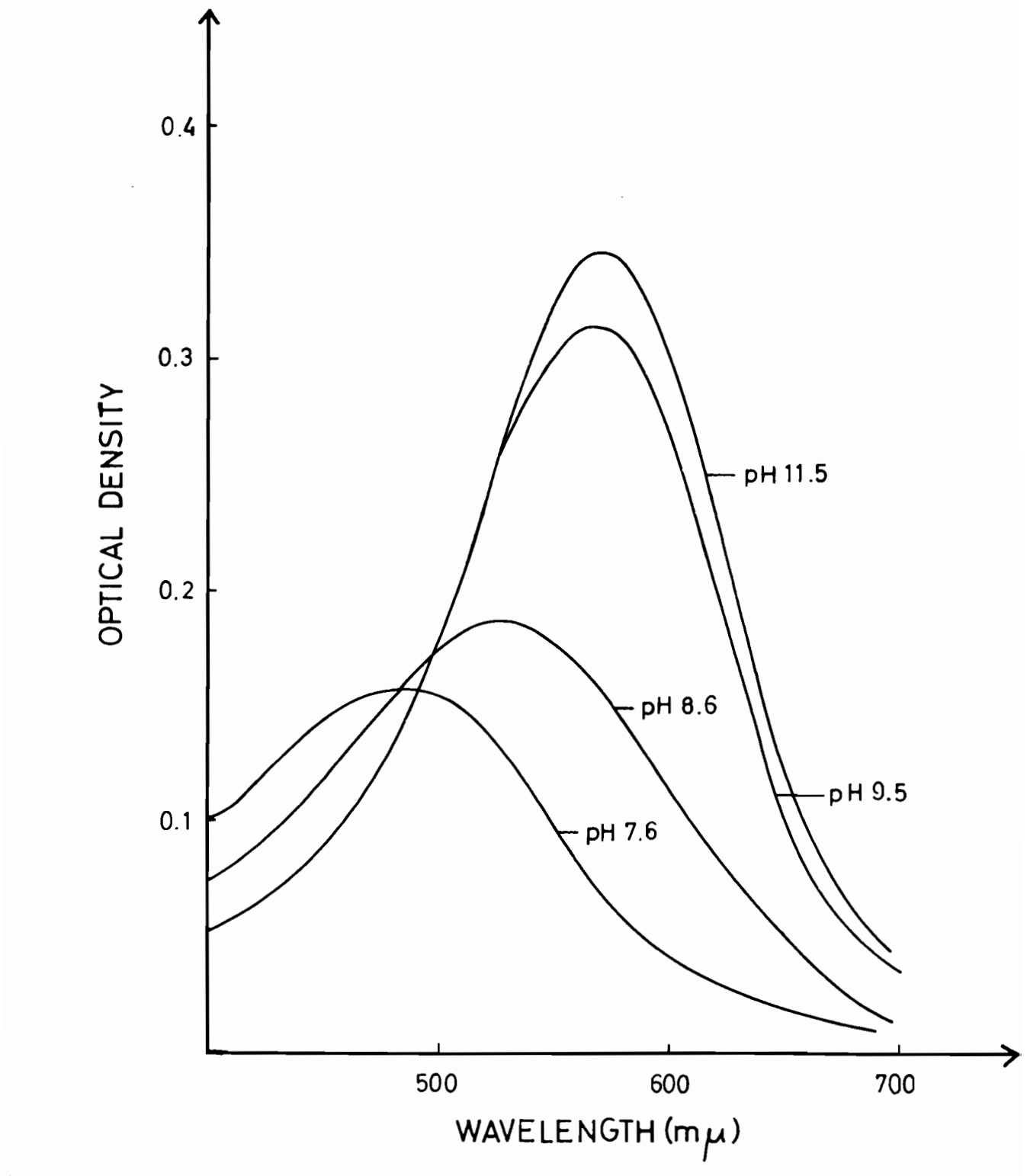

0.4
0.3
0.2
0.1
OPTICAL DENSITY
pH 11.5
pH 8.6
pH 9.5
pH 7.6
500
600
700
WAVELENGTH (mμ)

FIGURE 5

The effect of pH on the absorption spectrum of a 1 x 10^{-5}M solution of NS-R'.

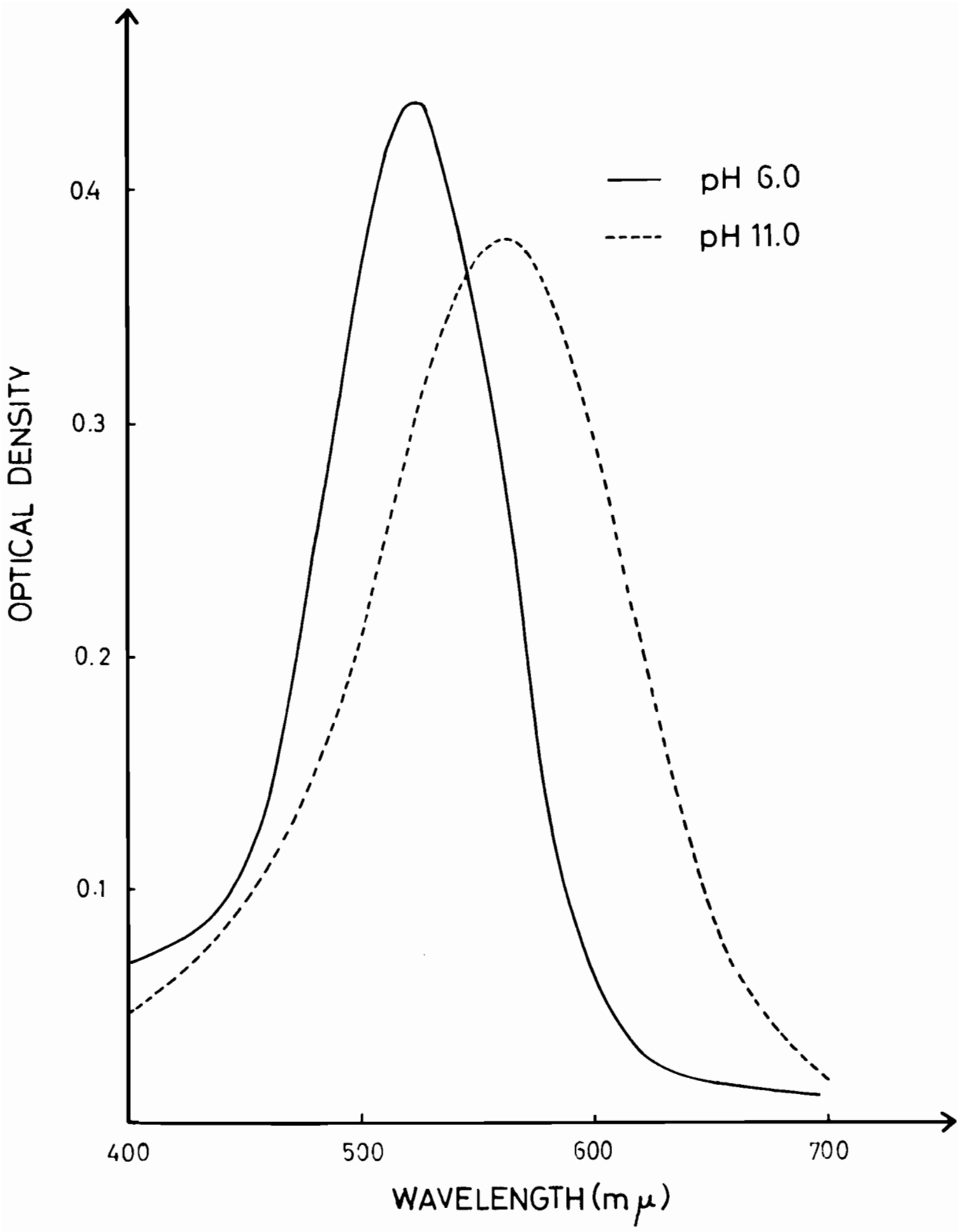

pH 6.0
pH 11.0
OPTICAL DENSITY
0.4
0.3
0.2
0.1
400
500
600
700
WAVELENGTH (mμ)

FIGURE 6

Spectrophotometric titration curve of N-R'.

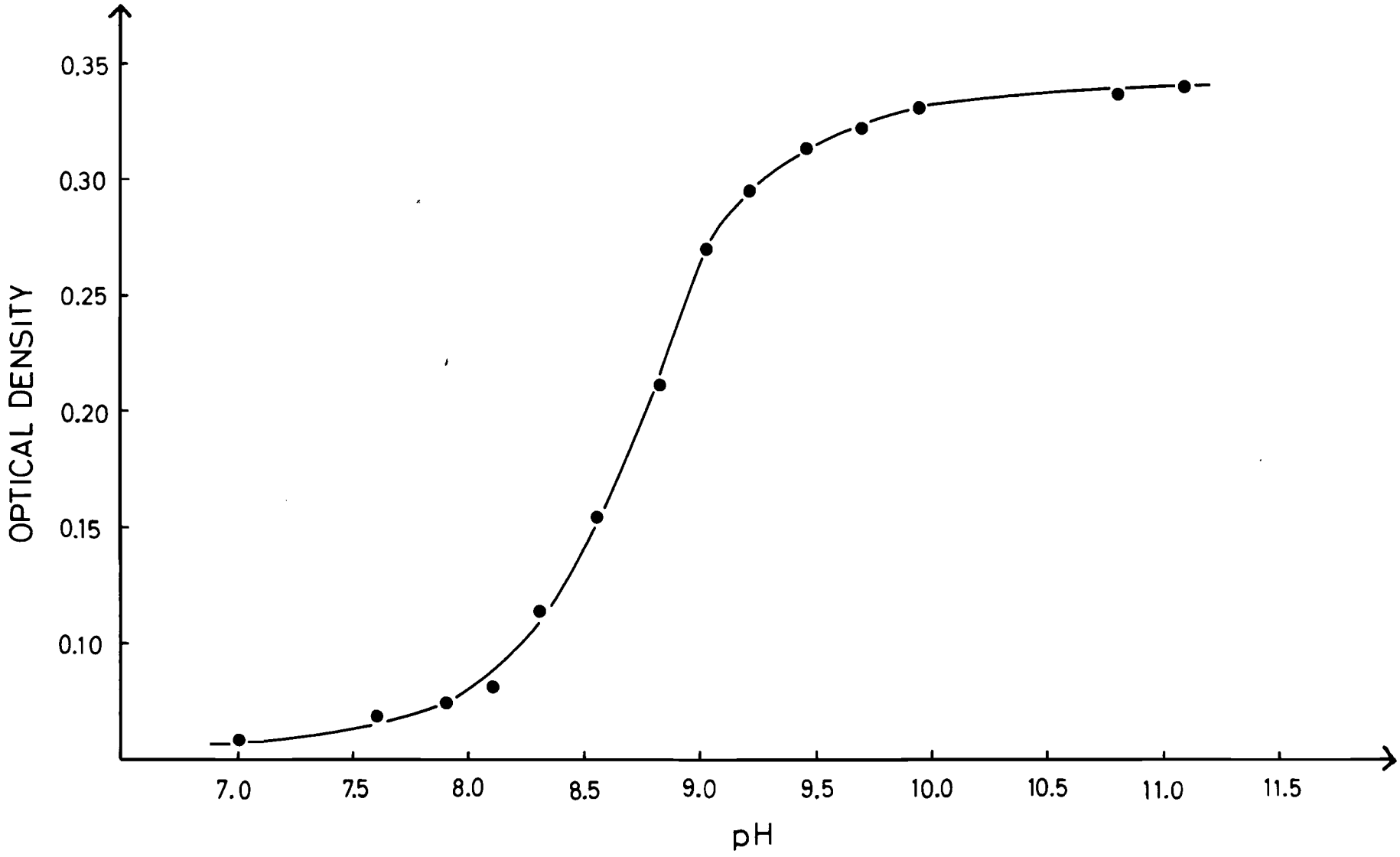
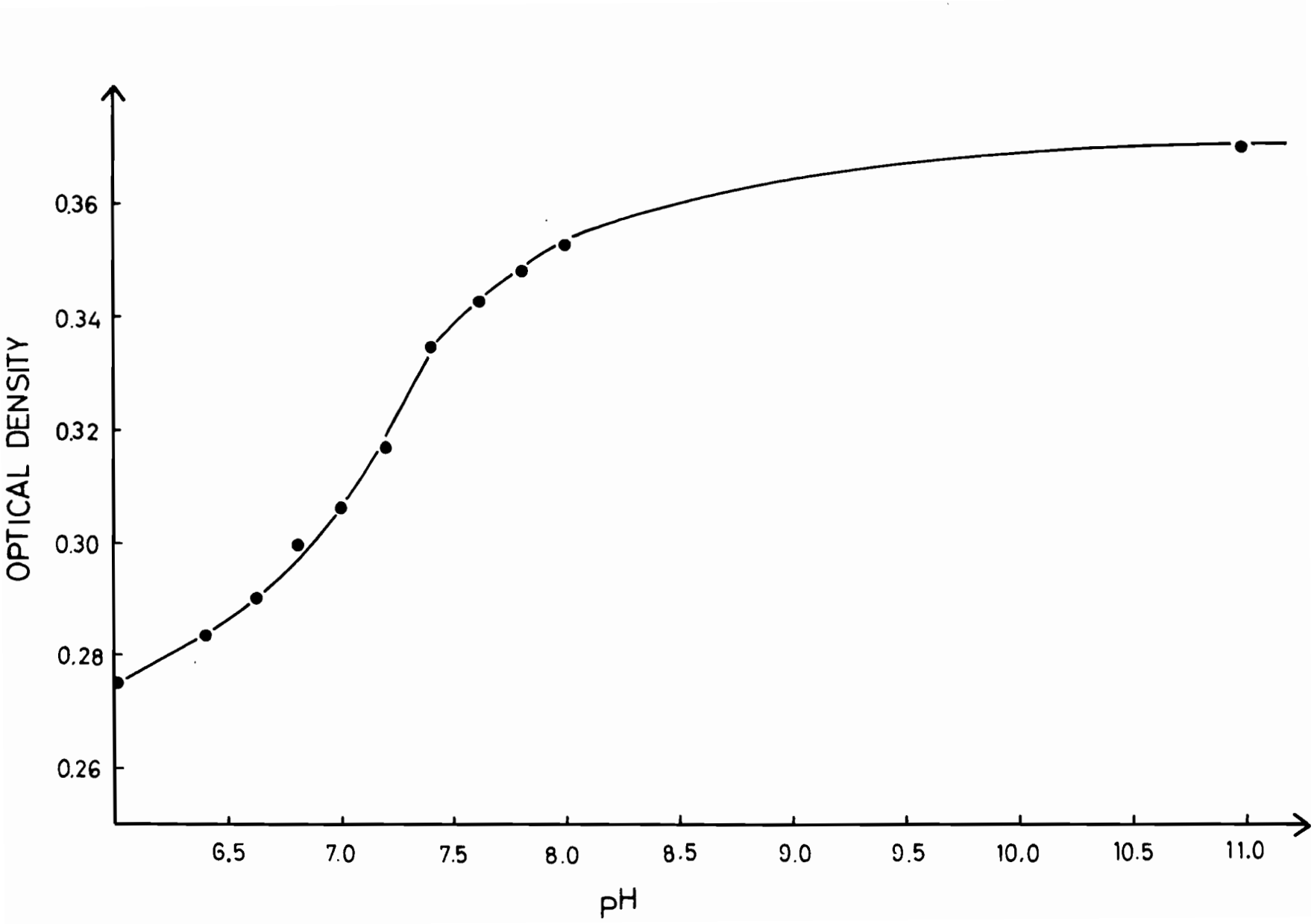

OPTICAL DENSITY
0.35
0.30
0.25
0.20
0.15
0.10
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
11.5
pH

FIGURE 7

Spectrophotometric titration curve of NS-R'.


OPTICAL DENSITY
0.36
0.34
0.32
0.30
0.28
0.26
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
pH

Proteins

Crystalline bovine serum albumin (BSA) was obtained from Armour Pharmaceutical Co., Kankakee, Ill. Optical density measurements of different BSA solutions were made at 280 mμ and the concentrations of each of the solutions was determined by micro-Kjeldahl analysis. From these data the extinction coefficient, $E^{1\%}_{1\ cm}$, was calculated as 6.8, and this value was used for the determination of the concentrations of BSA solutions. For the calculation of the molar concentrations the molecular weight of BSA was taken as 69,000 (163).

Anti-R antibodies were prepared by immunization of rabbits as described in ChapterV. The antibodies were isolated from decomplemented antisera by the fractionation procedure of Marrack et al (159), as recommended for the preparation of gamma globulin fractions. Antibody concentrations were determined with HSA-R conjugates from the appropriate precipitin curves. The molecular weight of rabbit antibodies was taken as 160,000 (15).

The ionic strength of all solutions was 0.1 and the buffers used were:

(i)	borate-KNO_3,	pH 8.0, 8.5, 9.0
(ii)	borate-NaCl,	pH 8.0
(iii)	phosphate	pH 7.0
(iv)	"Tris"*-NaCl	pH 7.0, 7.5
(v)	"Tris"-KNO_3	pH 8.0

* "Tris" refers to Tris(hydroxymethyl)-aminomethane.

THE TEMPERATURE-JUMP APPARATUS

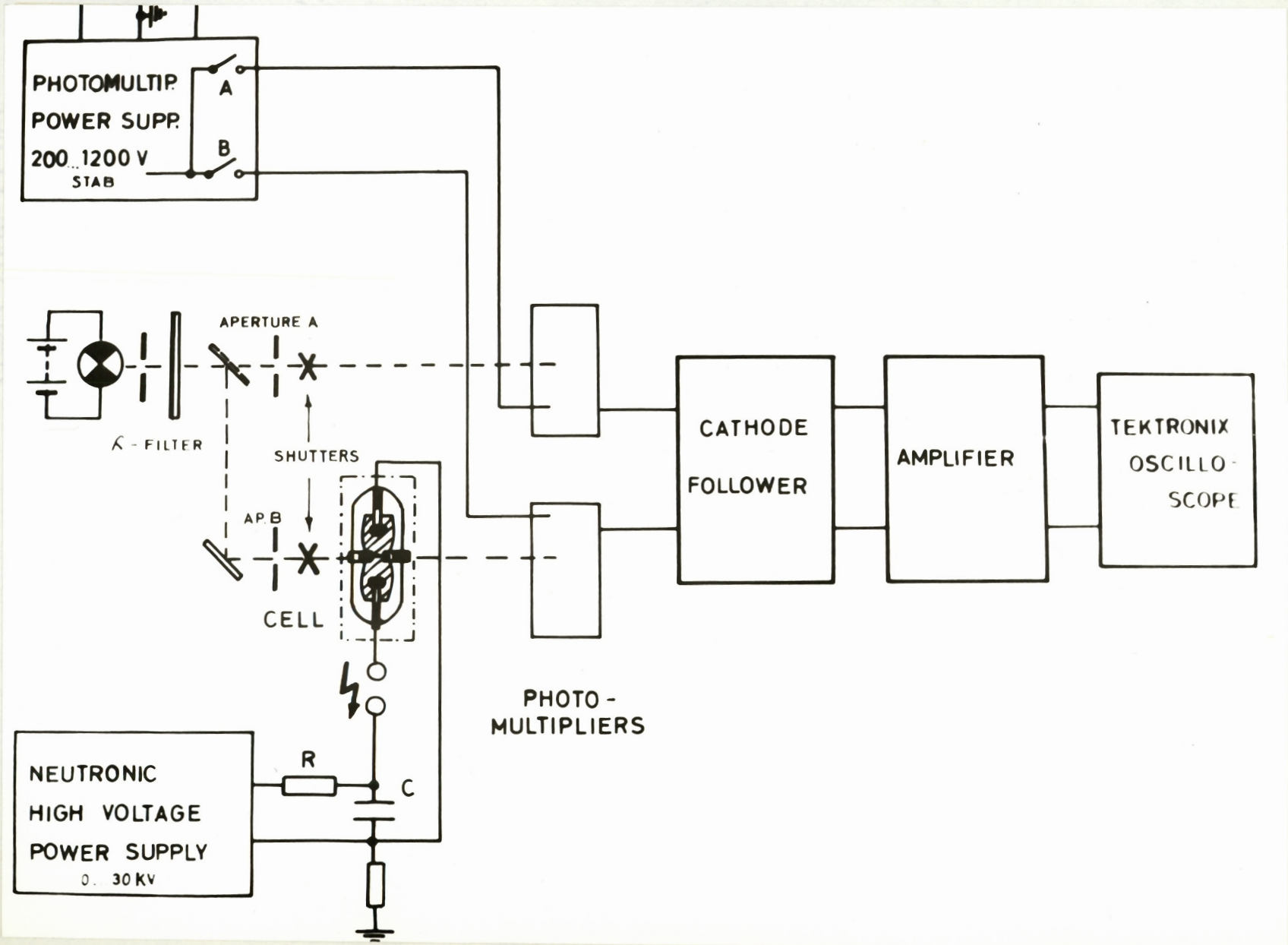
A detailed description of the apparatus used has been given elsewhere (138, 139). The general outline of this apparatus is given in Figs. 8 and 9.

The high voltage power supply (5-30 kV) obtained from Neutronic Associates, (Hempstead, N.Y.) was connected through a 400 mega-ohm resistor to a 0.1μF capacitor (Hydrawerke, Berlin). This capacitor was in turn connected to the reaction cell through a variable spark gap. The cell (Figs. 10 and 11) was built of sturdy plexiglass, able to withstand pressures of about 50 atmospheres. The electrodes (platinum or gold plated) were separated by a distance of 11 mm. For spectrophotometric observations, two quartz rods, separated by 1 cm, were cemented into the cell at right angles to the electrodes, and on opposite sides of the cell. Thus, the volume of the space enclosed by electrodes and quartz rods was about 1 cm^3, while the total volume of the cell was 50 cm^3. The cell itself was surrounded by an insulated jacket through which liquid from a thermostated bath was circulated. The whole cell assembly was contained in a light-tight housing. Both the cell and the condensor housing were shielded with Mu-metal in order to prevent electromagnetic waves from disturbing the sensitive electronic detection devices.

The changes in concentration were measured spectrophotometrically. The light source consisted of either a

FIGURE 8

Block 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

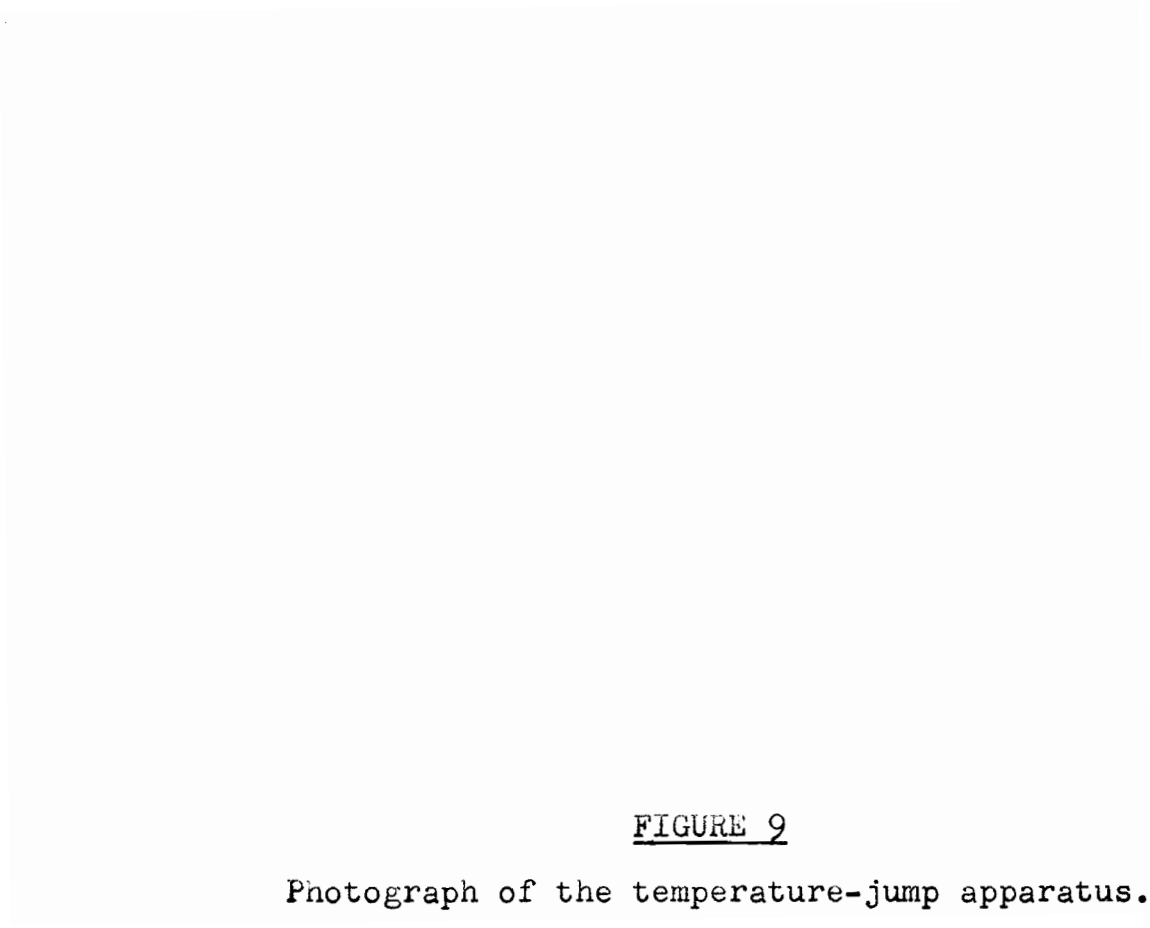


FIGURE 9

Photograph of the temperature-jump apparatus.

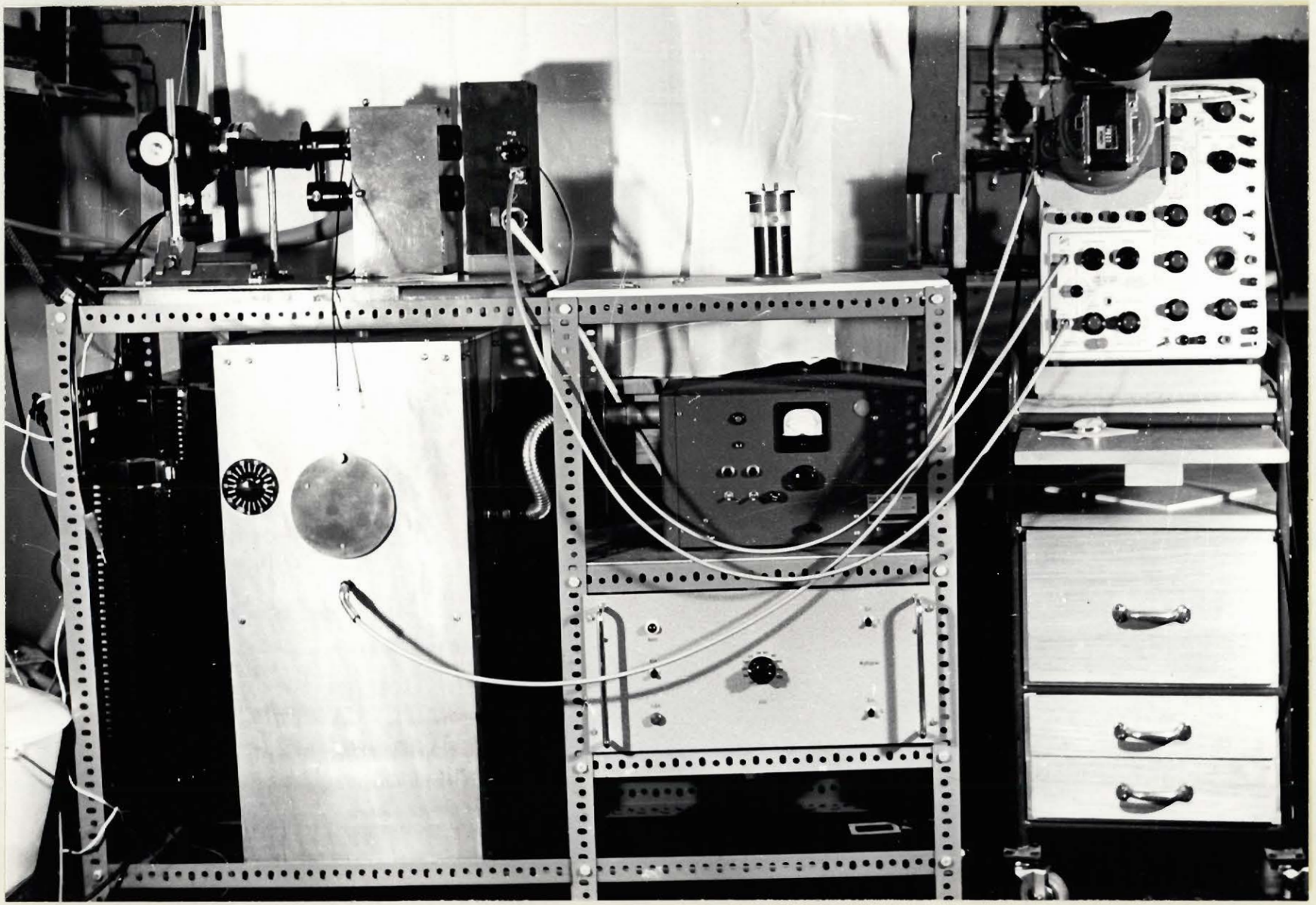


FIGURE 10

Cross-section of the temperature-jump cell.

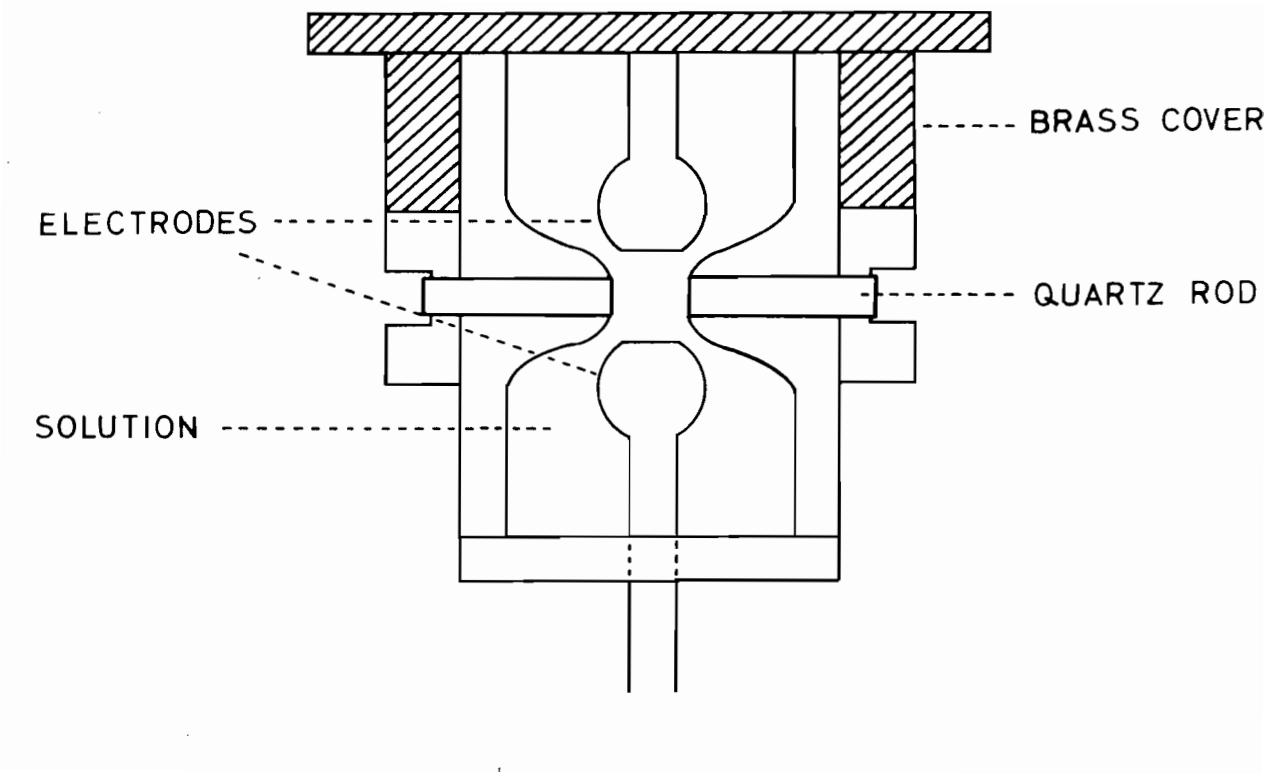
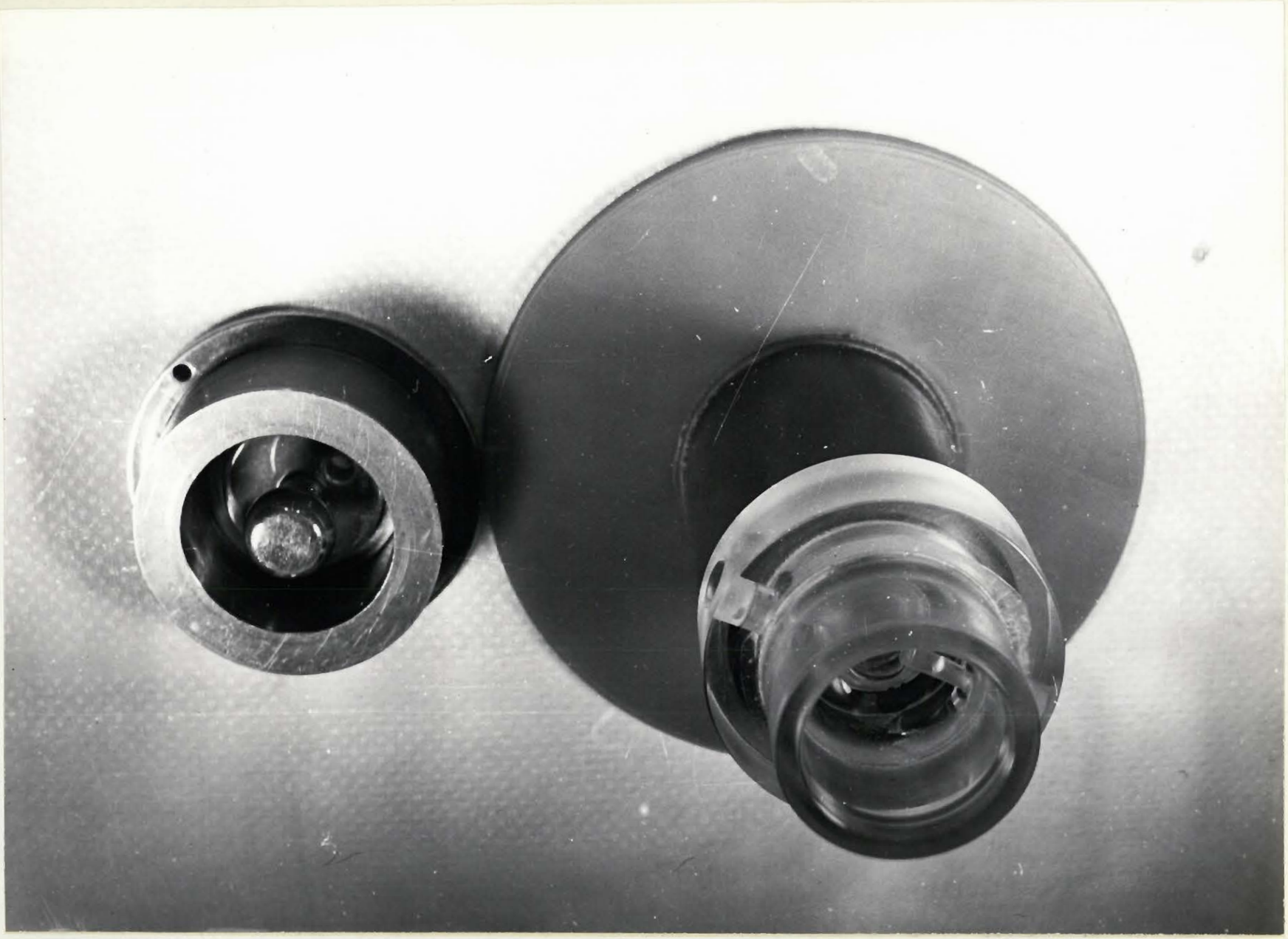

BRASS COVER
ELECTRODES
QUARTZ ROD
SOLUTION

FIGURE 11

Photograph of the temperature-jump cell.



tungsten filament lamp (Osram, 100 watt) or a mercury high pressure lamp (Osram, HBO 200), and was used in conjunction with an interference filter (a monochromator can also be used). The filtered light beam was split into two beams by a half-silvered mirror. One beam was made to pass through the cell and the other, the reference beam, passed through air. The intensities of the two beams were measured by two photo-multipliers (RCA 1P28), which were operated by a stabilized power supply, designed and built by Dr. L. de Maeyer at the "Max-Planck-Institut für physikalische Chemie" at Göttingen, Germany.

The signal from the two photomultipliers was amplified by a Tektronix plug-in unit (Type 53/54D) and was projected on a Tektronix oscilloscope screen.

The sequence of operations during an experiment may be described as follows: The cell containing the solution to be studied was placed into the thermostated jacket and allowed to attain the temperature of the thermostat. The dark currents of the two photomultiplier units were balanced against each other by means of a slide wire resistance, which was connected to the two cathode followers. The light source was turned on and the intensities of the main and reference beams, falling on their respective photomultipliers, were balanced against each other by means of iris diaphragms. The condensor was then charged slowly through the 400 mega-ohm resistor to a voltage

of about 30 kV. At this point the condenser discharged through the spark gap, raising the temperature of the solution between the electrodes by about 10°C within approximately 5 microseconds. The electromagnetic waves, generated by the spark, triggered off the horizontal sweep of the oscilloscope, which could be set for sweep rates from 1 μsec to 1 sec per cm on the screen.

The change of the intensity of the light beam passing through the reaction mixture, was followed by the vertical deflection of the electron beam on the oscilloscope screen as a function of time. The relaxation pattern obtained was recorded photographically, using a 35 mm "Robot" camera.

The heat generated in the reaction zone between the electrodes was dissipated by convection into the bulk of the solution, and the solution in the reaction zone, i.e. within the space between the electrodes, was also equilibrated with the bulk of the solution through convection. This process of re-equilibration required about 2 minutes. By the end of this time a new discharge could be triggered.

This procedure could be repeated as many times as desired, provided the reactants were not destroyed by processes occurring at the electrodes, or by the rise in temperature.

METHODS

Determination of the extent of the binding of the dyes to bovine serum albumin and anti-R antibodies

Since a spectral shift towards longer wavelengths occurred on combination of the dyes with BSA or with anti-R

antibodies, the extent of this binding was determined spectrophotometrically by the method described by Klotz (113) and Westphal (165). If the molar extinction coefficients of the bound and free forms of the dyes are known, the fraction of free dye can be calculated from the relationship

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

where ϵ_{app} is the apparent molar extinction coefficient of the dye, and ϵ_f and ϵ_b are the molar extinction coefficients of the free and bound forms of the dye, respectively.

To evaluate ϵ_b, at a wavelength of 610 mμ, the apparent molar extinction coefficients were determined for a series of solutions containing a constant amount of dye and varying amounts of BSA or of the specific antibodies. Protein solutions of appropriate concentrations and in the absence of the dyes were used as blanks in the spectrophotometric measurements. The values of ϵ_{app} were then plotted against the different ratios of dye/protein concentrations, and ϵ_b was identified with the molar extinction coefficient of the solution at infinite protein concentration. All spectrophotometric measurements were made in a Zeiss spectrophotometer.

The number of binding sites on the BSA molecule was calculated with the help of the equation

$$\frac{1}{b} = \frac{1}{K(P)c} + \frac{1}{(P)}$$

which was used previously by Nisonoff and Pressman (115) for

antibody-hapten systems*. In this equation b and c represent the molar concentrations of bound and free dye, respectively, P is the total molar concentration of the binding sites of the protein, and K is the equilibrium constant. For this purpose varying amounts of dye were added to solutions containing a constant amount of BSA and the concentrations of bound and free dye were calculated from optical density measurements.

Relaxation time measurements

Relaxation time measurements were made with the apparatus described in a preceding section. Solutions containing varying concentrations of protein and dye were prepared in appropriate buffer solutions. The final ionic strength of these solutions was always 0.1. The equilibrium concentrations of the bound and free dye at the temperature corresponding to that reached after discharge, i.e. values of $\bar{c}$, were calculated from optical density measurements at a wavelength of 610 mμ in a Zeiss spectrophotometer. The rate of re-equilibration at this higher temperature was followed on the oscilloscope. The wavelength of the beam traversing the cell was 590 mμ, because of the higher sensitivity of the photomultipliers at this wavelength. For each solution several relaxation curves were obtained, using different sweep-rates of the horizontal beam of the oscilloscope. The relaxation time, τ, was calculated

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* This relationship is essentially similar to the equation given on page 26 in Chapter III.

from each relaxation curve and the average value was computed.

In early kinetic experiments a tungsten filament lamp was used as the light source. However, due to its low intensity, only small relaxation effects were observed. Later a mercury high pressure lamp was employed, because of its higher intensity. The disadvantage of the mercury lamp was its instability, which led to intensity fluctuations. These intensity fluctuations occurred within the time range of the relaxation effects due to protein-dye interactions and rendered some of the relaxation measurements uncertain.

RESULTS AND DISCUSSION

(a) BSA-DYE INTERACTIONS

Spectral shifts of the dyes

The rationale for synthesizing the particular dyes, N-R' and NS-R', was that these compounds incorporated the antigenic determinant, i.e. the phenyl arsonate group, and that they had similar structures to that of Orange I, which is a pH indicator. As mentioned earlier, the absorption spectra of both dyes were shifted to longer wavelengths in absence of proteins in solutions of increasing pH. Spectral shifts in the same direction were observed when BSA was added to both dyes. Therefore, to obtain maximum spectral shifts due to BSA-dye interactions, most of these studies were done in buffers having pH values which were lower than the pK values of the respective dyes, i.e. below pH 8.7 for N-R' and below pH 7.2 for NS-R'.

The effects of BSA on the absorption spectrum of N-R' in borate-KNO_3 buffer (pH 8.0, $\Gamma/2 = 0.1$) and in "Tris"-KNO_3 buffer (pH 8.0, $\Gamma/2 = 0.1$) are shown in Figs. 12 and 13, respectively. It is evident that the absorption maximum was shifted from 510 mμ for the free dye, to 610 mμ for the bound dye. A shift to longer wavelength was also observed for the bound dye at pH 7.0 in phosphate buffer (Fig. 14). As can be seen from Fig. 13, however, this spectral shift was smaller than that obtained with N-R'.

FIGURE 12

The effect of BSA on the absorption spectrum of a 9.1×10^{-6}M solution of N-R' in borate-KNO_3 buffer (pH 8.0, $\Gamma/2 = 0.1$).

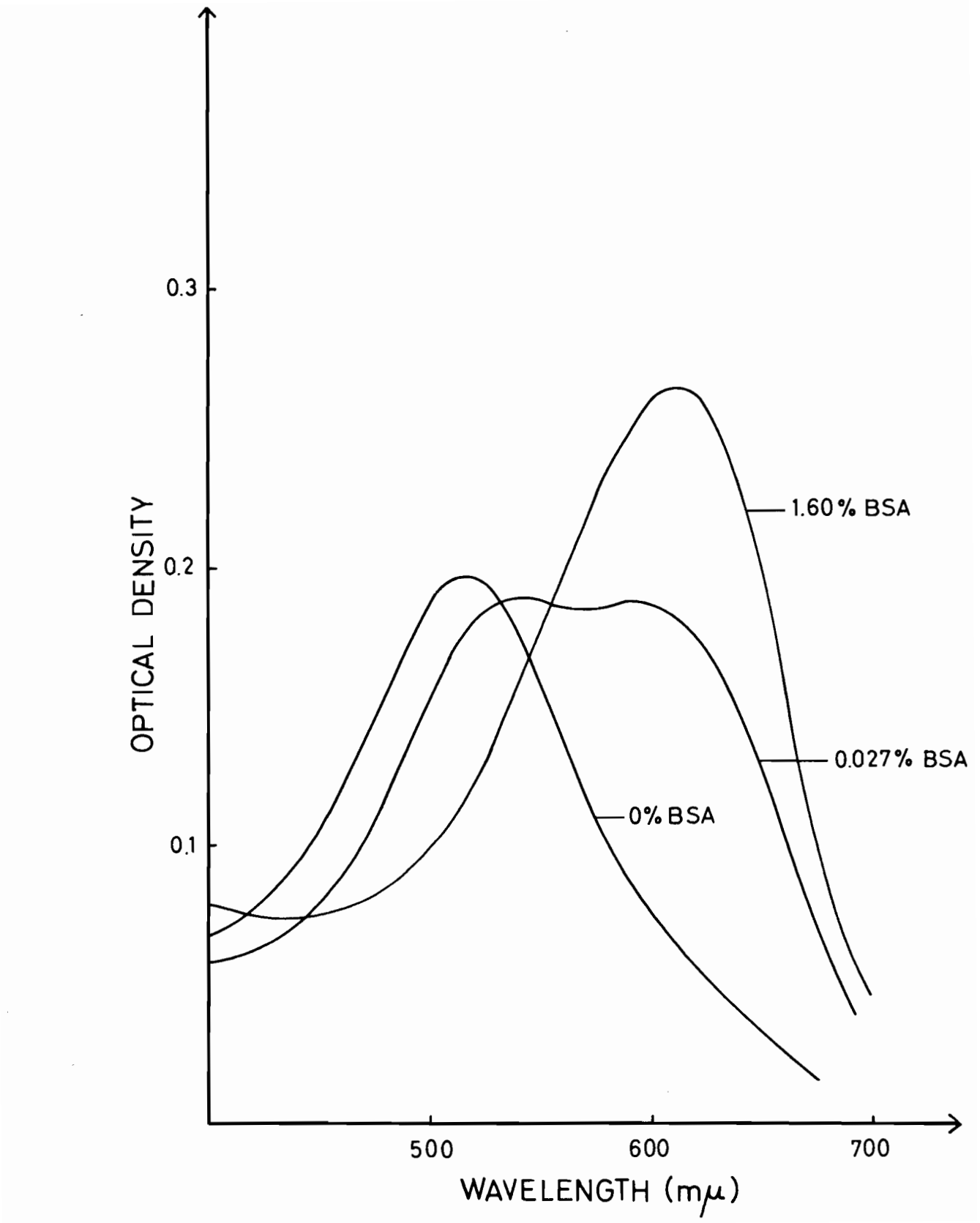

0.3
0.2
0.1
OPTICAL DENSITY
1.60% BSA
0.027% BSA
0% BSA
500
600
700
WAVELENGTH (mμ)

FIGURE 13

The effect of BSA on the absorption spectrum of a 9.1 x 10^{-6}M solution of N-R' in "Tris"-KNO_3 buffer (pH 8.0, $\Gamma/2 = 0.1$).

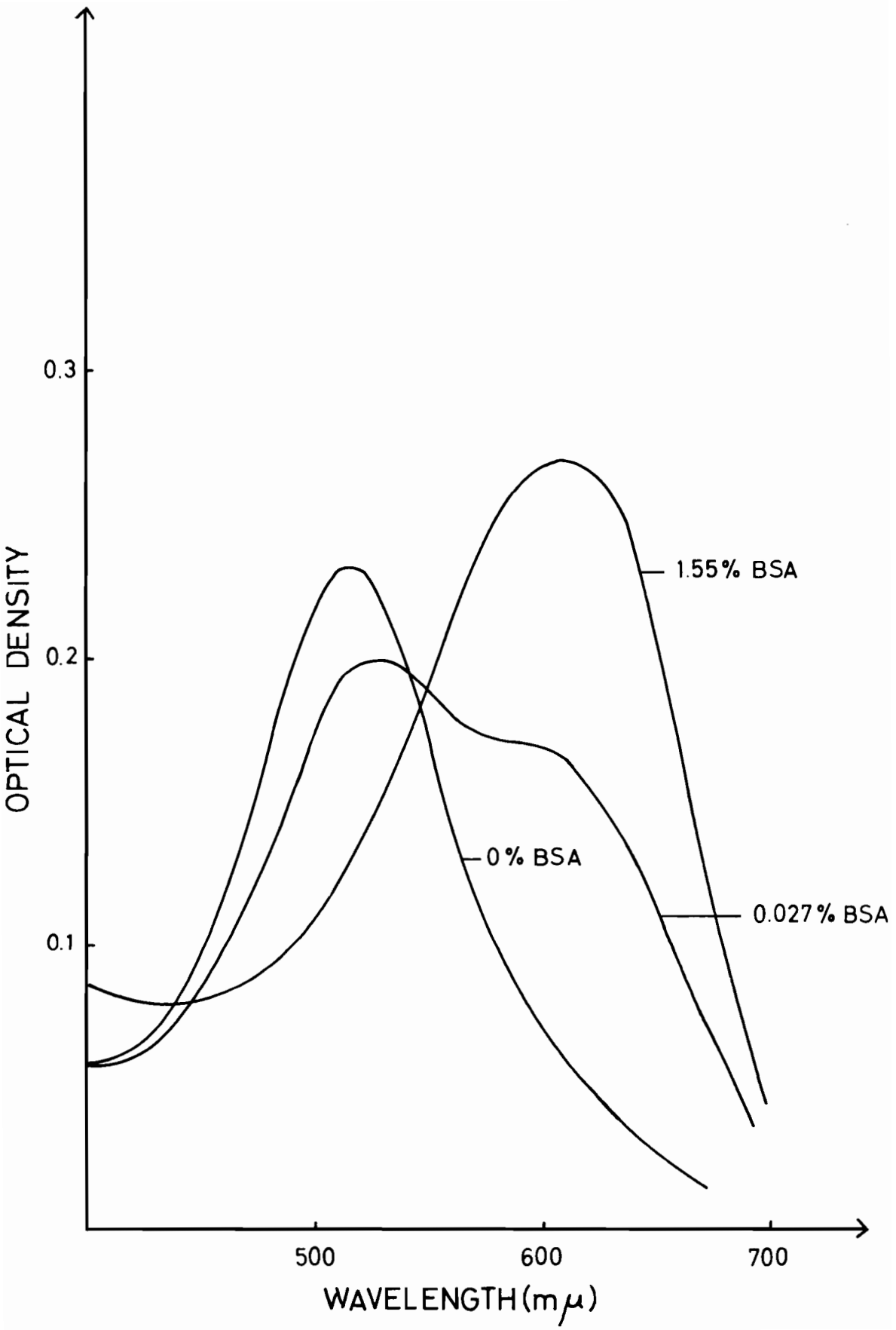
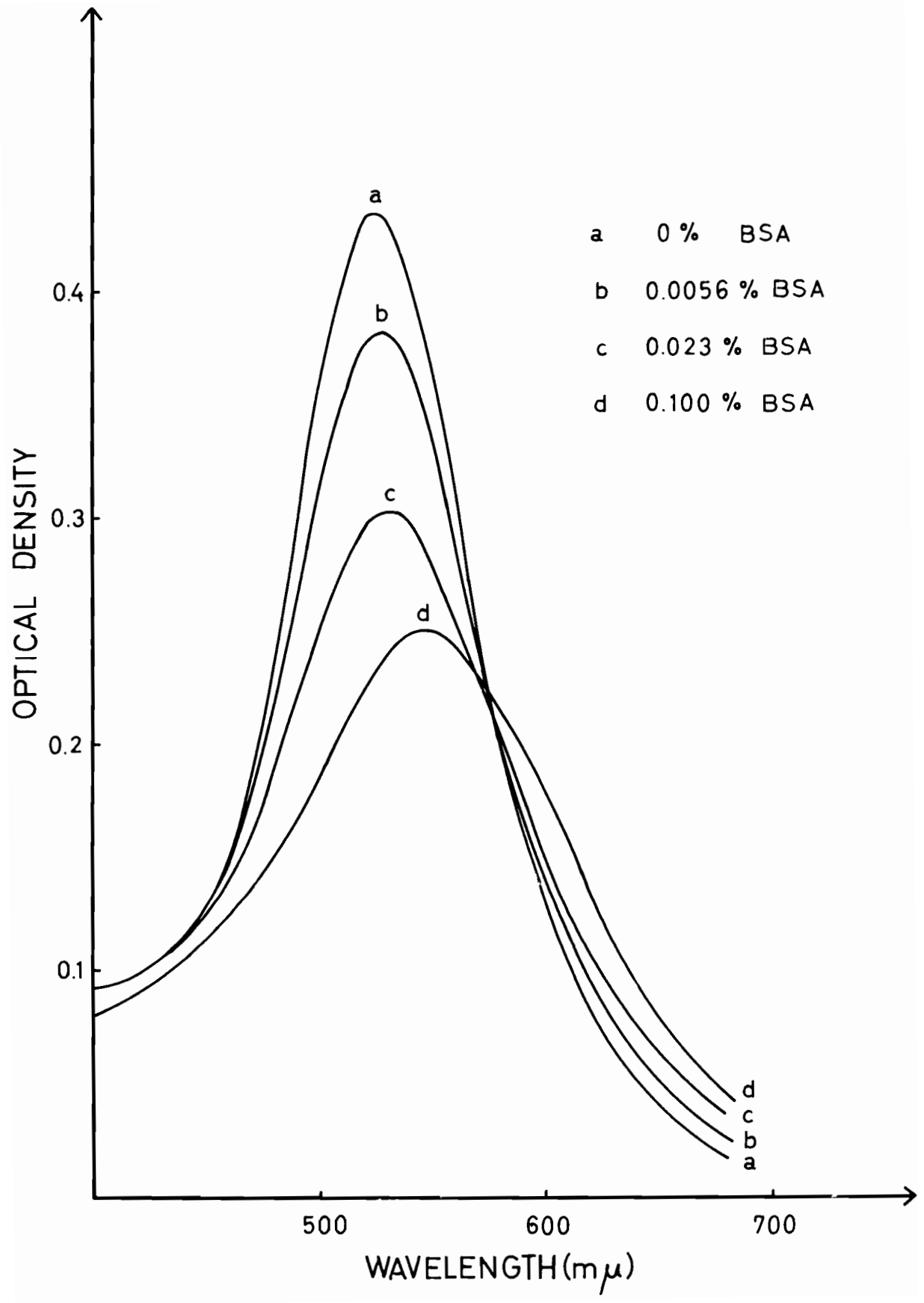

OPTICAL DENSITY
0.3
0.2
0.1
1.55% BSA
0% BSA
0.027% BSA
500
600
700
WAVELENGTH (mμ)

FIGURE 14

The effect of BSA on the absorption spectrum of a 9.1×10^{-6}M solution of NS-R' in phosphate buffer (pH 7.0, $\mu/2 = 0.1$).


a 0 % BSA
b 0.0056 % BSA
c 0.023 % BSA
d 0.100 % BSA
OPTICAL DENSITY
0.4
0.3
0.2
0.1
500
600
700
WAVELENGTH (mμ)
a
b
c
d
d
c
b
a

Attempts to study the binding between NS-R' and BSA at pH 6.5 were not successful, since no spectral shift was observed at this pH.

The spectral shifts to longer wavelengths, caused by the interaction of the dyes with BSA, are attributed to a lowering of the pK values of these dyes. This effect is considered to favour delocalization of the electrons in the dye molecules, which results in a larger contribution of the quinonoid form to the resonance structures of the dyes.

To study the kinetics of a reversible system by the temperature-jump method, it is necessary to determine the concentrations of free and bound reactants at equilibrium. Usually, for protein-dye interactions, this can be achieved by equilibrium dialysis. However, in the present study the use of this method had to be ruled out because of the high affinities of these dyes for the dialysis (Visking) membrane. Therefore, equilibrium data were obtained by the spectrophotometric technique, described previously. For this purpose the extinction coefficients of the bound and free forms for each of the two dyes were obtained at a wavelength at which - preferably - the difference between the two extinction coefficients was largest.

As can be seen from Figs. 12 and 13, the optical density at the wavelength of maximum absorption for the bound form of the N-R' dye, in borate and "Tris" buffers, respectively, increased steadily with increasing amounts of BSA. However,

some irregularities were observed in the wavelength region corresponding to the maximum absorption of the free dye, i.e. at 510 mμ, in as much as the optical density increased at first, in the presence of small amounts of BSA, and decreased with increasing concentrations of BSA*. No simple explanation can be offered for this effect, however, it is conceivable that this effect might be due to the change in the dielectric constant of the solution caused by the presence of the various buffer ions and of BSA. On the other hand, NS-R' exhibited a regular decrease in optical density at 530 mμ which corresponded to the wavelength of maximum absorption for the free form and a regular increase at 610 mμ, which was attributed to the bound form of the dye.

Determinations of ϵ_b at 610 mμ for the dyes N-R' and NS-R', in borate buffer (pH 8.0, $\Gamma/2 = 0.1$) and in phosphate buffer (pH 7, $\Gamma/2 = 0.1$), are shown in Figs. 15 and 16, respectively. In these figures values of ϵ_{app} were plotted against different ratios of dye/BSA, and ϵ_b was obtained by extrapolation of the curves to infinite BSA concentrations. The following molar extinction coefficients were determined.

N-R': $\epsilon_b = 2.80 \times 10^4\ M^{-1}cm^{-1}$, $\epsilon_f = 0.58 \times 10^4\ M^{-1}cm^{-1}$

NS-R': $\epsilon_b = 2.10 \times 10^4\ M^{-1}cm^{-1}$, $\epsilon_f = 1.04 \times 10^4\ M^{-1}cm^{-1}$

Since the difference in extinction coefficients at 610 mμ for

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* These conclusions are based on absorption curves obtained with 10 different BSA concentrations, however, to simplify the presentation of these results, only a few typical absorption curves are given in Figs. 12 and 13.

FIGURE 15

Determination of ϵ_b of N-R', bound by BSA, for two different dye concentrations in borate-KNO_3 buffer (pH 8.0, $\Gamma/2 = 0.1$).

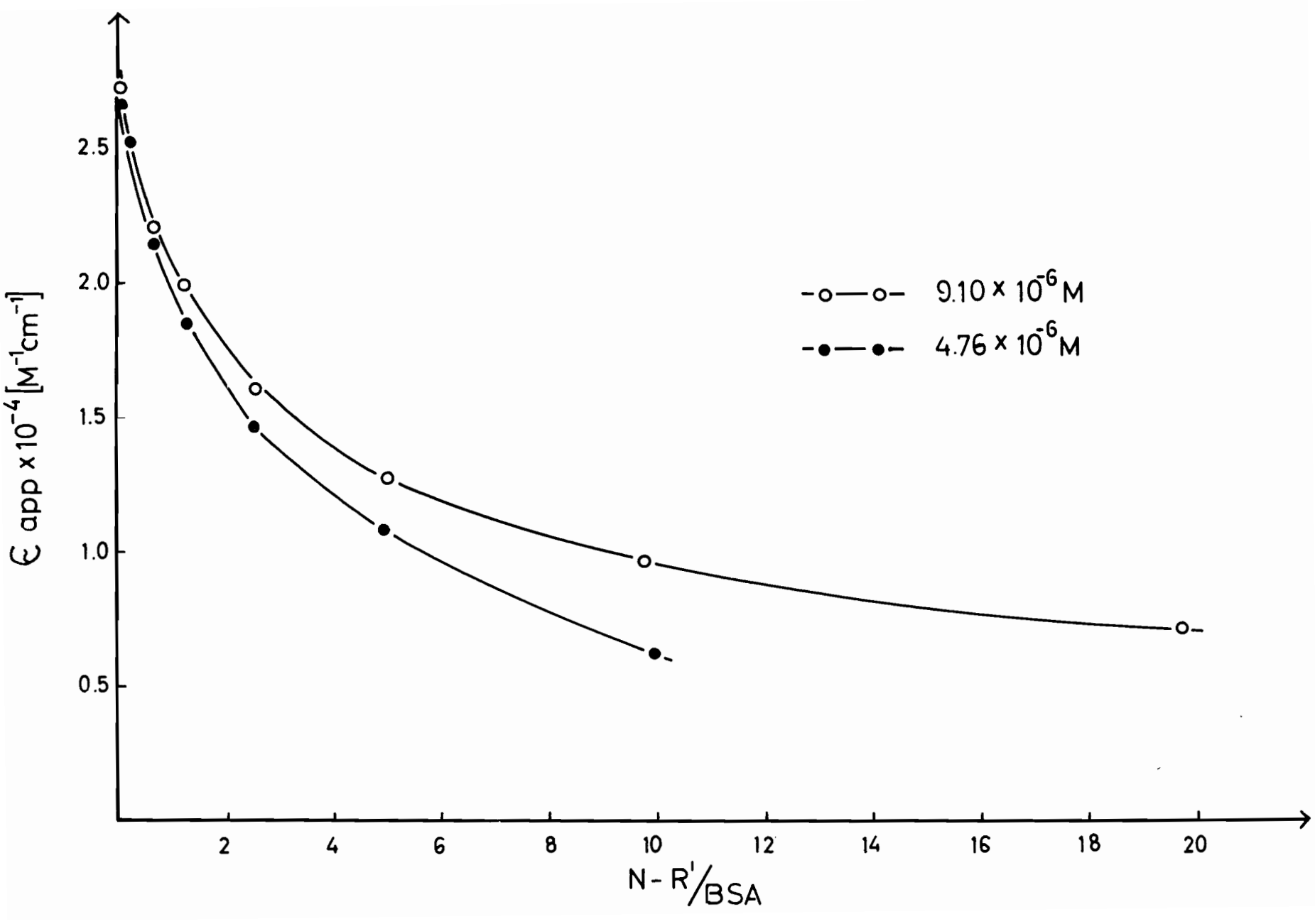
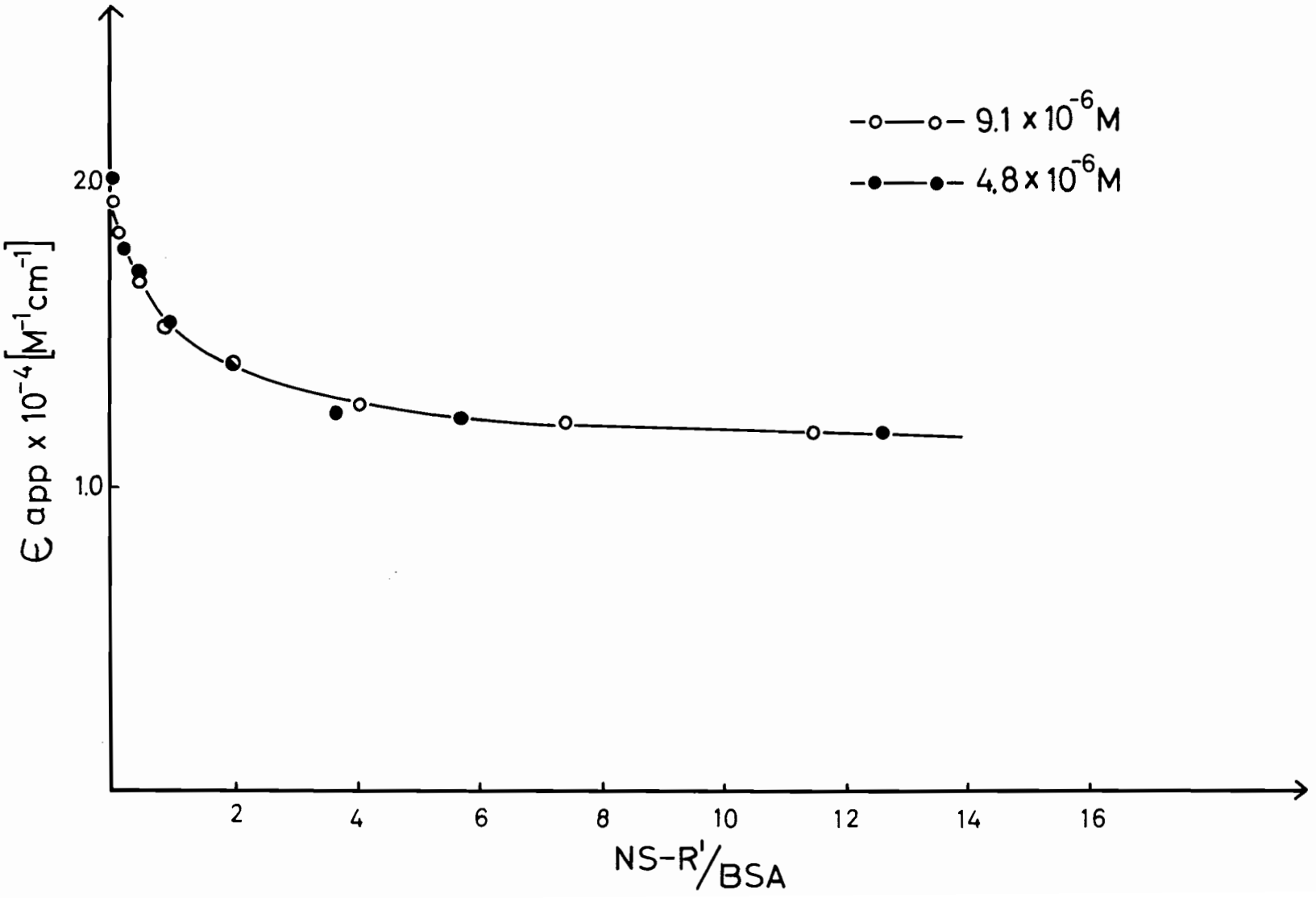

2.5
2.0
1.5
1.0
0.5
ε app x 10⁻⁴ [M⁻¹cm⁻¹]
9.10 x 10⁻⁶ M
4.76 x 10⁻⁶ M
2
4
6
8
10
12
14
16
18
20
N - R'/BSA

FIGURE 16

Determination of ϵ_b of NS-R', bound by BSA, for two different dye concentrations in phosphate buffer (pH 7.0, $\Gamma/2 = 0.1$).


-o—o- 9.1 x 10^-6 M
-●—●- 4.8 x 10^-6 M
2.0
1.0
ε app x 10^-4 [M^-1 cm^-1]
2
4
6
8
10
12
14
16
NS-R'/BSA

the system BSA:N-R' was much greater than that for BSA:NS-R', the former was used for most kinetic studies.

Equilibrium Experiments

The above extinction coefficients were used for the calculation of the extent of binding between BSA and the two dyes. The binding curve obtained with solutions containing 2.00×10^{-6} M BSA and varying concentrations of N-R' is shown in Fig. 17. The corresponding data are listed in Table VI. From these data, and on the basis of the relationship

$$\frac{1}{b} = \frac{1}{K(P)c} + \frac{1}{(P)}$$

the number of binding sites per molecule of BSA was calculated as 5. The non-linearity of the plot given in Fig. 16 indicates a heterogeneity of the binding constants of the different combining sites of the BSA molecule. A heterogeneity constant, a, of 0.6 was calculated according to the method of Nisonoff and Pressman (115), as described in Chapter III using the equation

$$\frac{1}{F_B} = \frac{1}{(K_o c)^a} + 1$$

The average equilibrium constant, K_o, for the association between N-R' molecules and BSA binding sites was thus computed as $1 \times 10^5 M^{-1}$.

The results for the interaction of NS-R' with BSA in phosphate buffer (pH 7.0, $\Gamma/2 = 0.1$) are given in Fig. 18. The straight line in Fig. 18 indicates that the BSA molecules did not exhibit any heterogeneity with respect to the binding of

FIGURE 17

The binding of N-R' by BSA in borate-KNO_3 buffer (pH 8.0, $\Gamma/2 = 0.1$) at 25°C. Here c and b represent the free and bound dye concentrations, respectively.

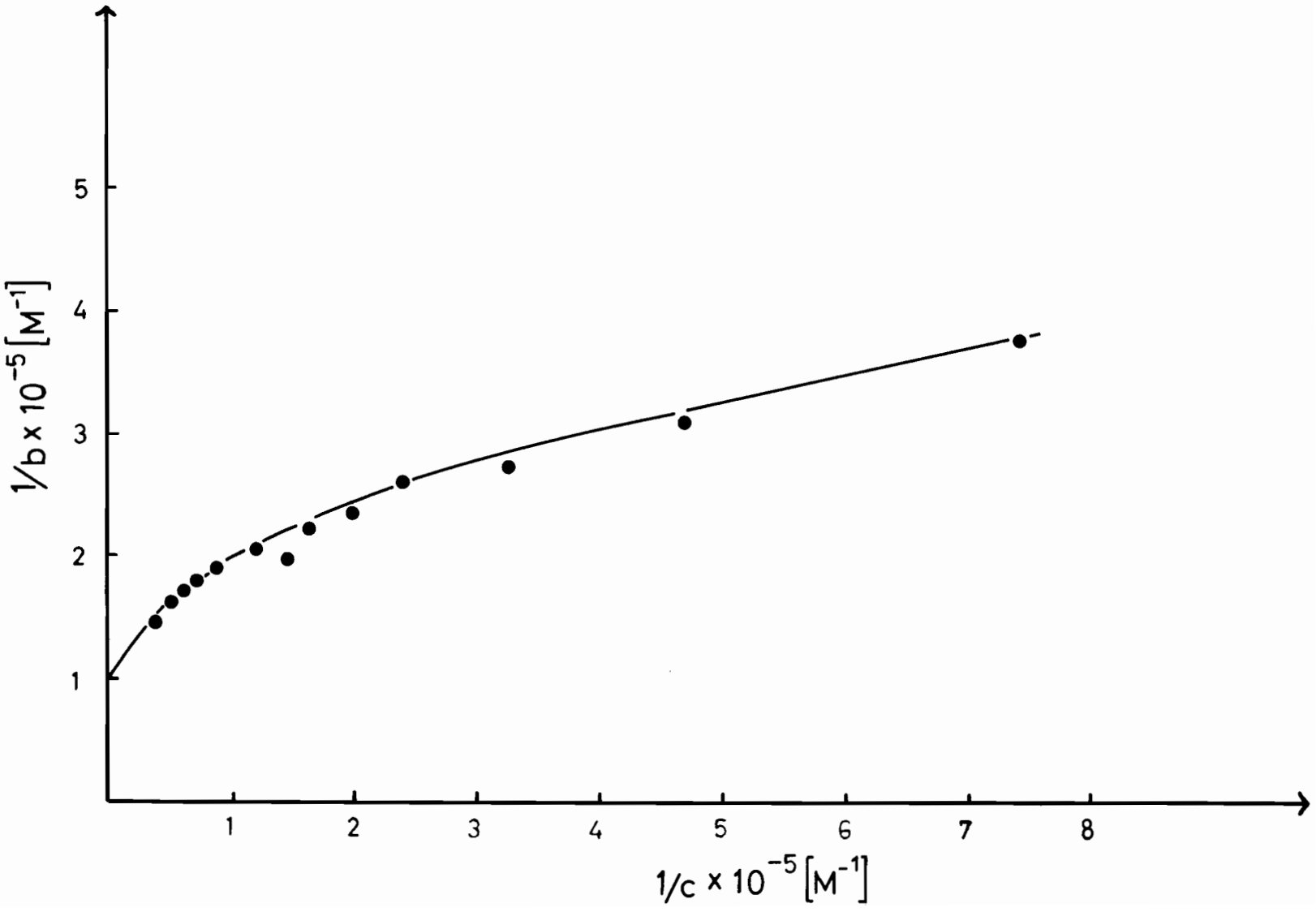

1/b x 10^-5 [M^-1]
5
4
3
2
1
1
2
3
4
5
6
7
8
1/c x 10^-5 [M^-1]

TABLE VI

Binding Data for the Reaction of N-R' with BSA

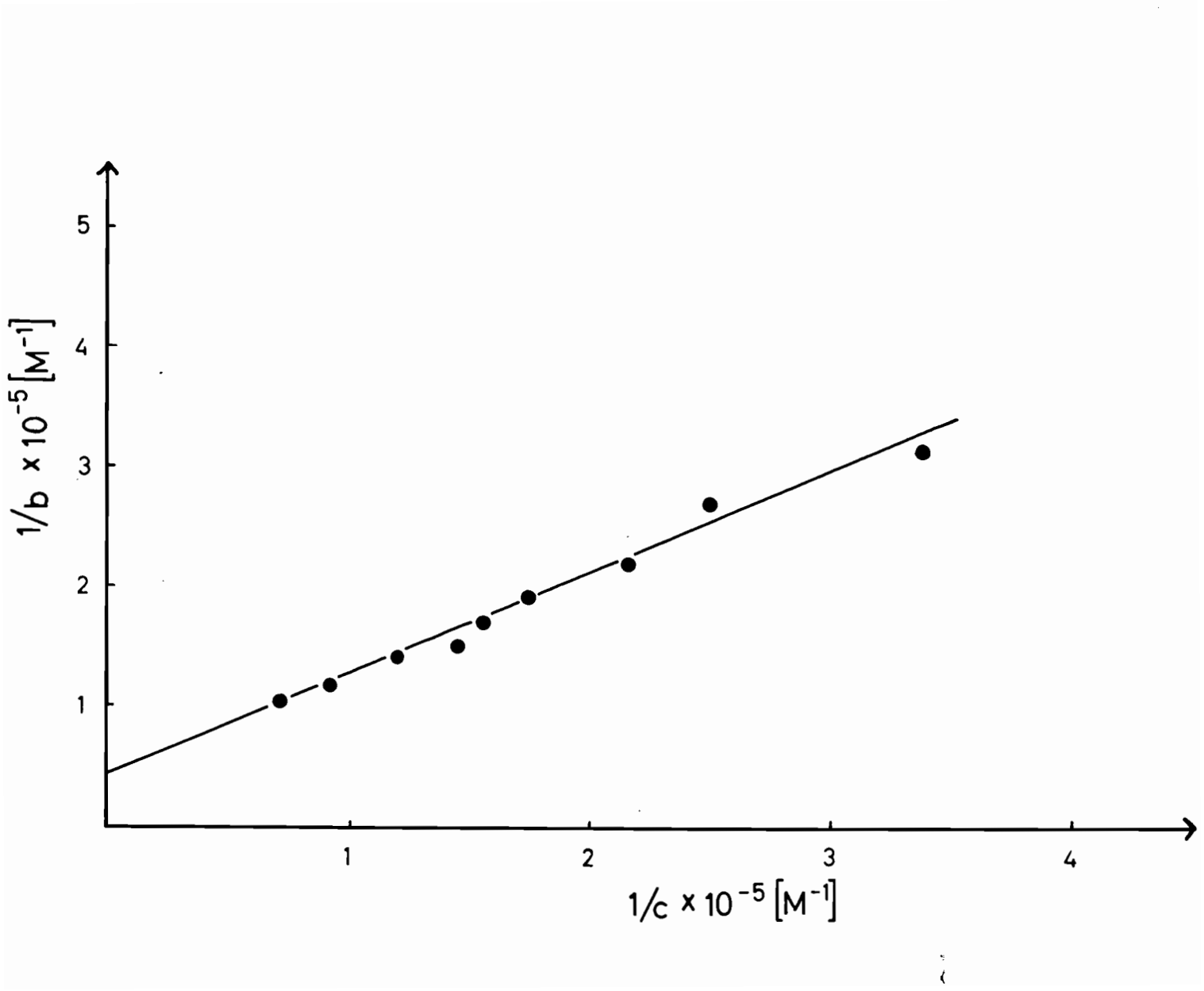
(N-R') x 10^5 [M]	Optical Density	ϵ_{app} x 10^{-4} [$M^{-1}cm^{-1}$]	α*	1 - α	c x 10^5 [M]	b x 10^5 [M]	1/c x 10^{-5} [M^{-1}]	1/b x 10^{-5} [M^{-1}]
0.400	0.082	2.05	0.337	0.663	0.135	0.265	7.41	3.78
0.533	0.102	1.91	0.400	0.600	0.213	0.320	4.70	3.13
0.667	0.119	1.78	0.459	0.541	0.306	0.361	3.27	2.77
0.800	0.131	1.64	0.522	0.478	0.418	0.383	2.39	2.61
0.933	0.149	1.60	0.541	0.459	0.504	0.427	1.99	2.34
1.07	0.160	1.50	0.586	0.414	0.625	0.442	1.60	2.26
1.20	0.181	1.51	0.582	0.418	0.697	0.502	1.44	1.99
1.33	0.184	1.38	0.639	0.361	0.850	0.481	1.18	2.06
1.67	0.212	1.27	0.689	0.311	1.15	0.520	0.87	1.93
2.00	0.239	1.20	0.721	0.279	1.44	0.557	0.69	1.80
2.34	0.261	1.12	0.756	0.244	1.77	0.571	0.57	1.75
2.67	0.288	1.08	0.775	0.225	2.07	0.601	0.48	1.66
3.00	0.308	1.03	0.797	0.203	2.40	0.615	0.42	1.63
3.34	0.329	1.03	0.797	0.203	2.66	0.678	0.38	1.48

* Here α represents the fraction of the free dye. Values of α were calculated with the help of the equation $\alpha = (\epsilon_{app} - \epsilon_b)/(\epsilon_f - \epsilon_b)$

The values of ϵ_b and ϵ_f used, were 2.80 x 10^4 $M^{-1}cm^{-1}$ and 0.58 x 10^4 $M^{-1}cm^{-1}$, respectively.

FIGURE 18

The binding of NS-R' by BSA in phosphate buffer (pH 7.0, $\Gamma/2 = 0.1$) at 25°C. Here c and b represent the free and bound hapten concentrations, respectively.


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

NS-R'. From this plot one binding site was calculated per molecule of BSA. The corresponding equilibrium constant was computed as $5.5 \times 10^4 M^{-1}$.

Relaxation Experiments

To simplify the evaluation of relaxation time measurements, it was assumed that all the binding sites of the BSA molecule were identical and independent. Typical relaxation curves for the reaction between N-R' and BSA are shown in Fig. 19. In all cases the curves were characterized by a single relaxation time as demonstrated by the linearity of a plot of $\log \Delta c'$ versus t. An analysis of the concentration dependence of the relaxation time, τ, revealed that the relation

$$\frac{1}{\tau} = k_{-1} + k_1\left[(\bar{P}) + (\bar{D})\right]$$

described the behaviour of the system. In this relation $(\bar{P})$ and $(\bar{D})$ represent the free equilibrium concentrations of the BSA sites and of the dye molecules, respectively. Accordingly, as shown in Chapter IV, it would appear that the overall mechanism for the reaction of BSA with N-R' may be represented by

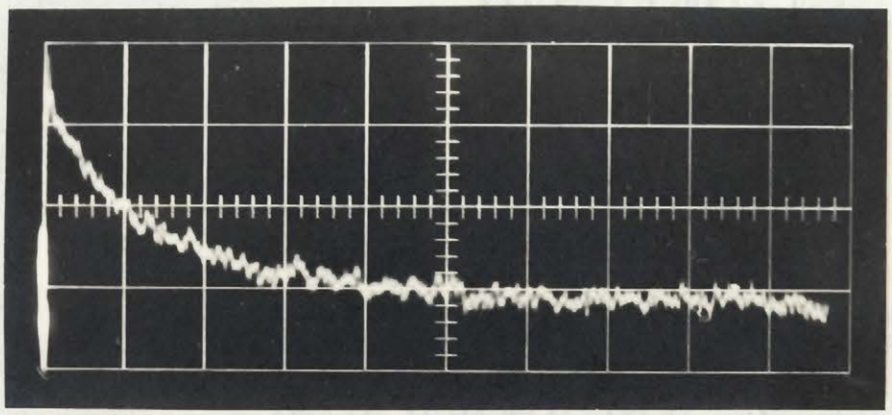
$$P + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PD$$

A typical set of data for the concentration dependence of τ for the interaction of N-R' with BSA in borate buffer (pH 8.0, $\Gamma/2 = 0.1$) at 25°C is given in Table VII. Plots of $1/\tau$ versus

FIGURE 19

Typical relaxation curves for the reaction between BSA and N-R'.

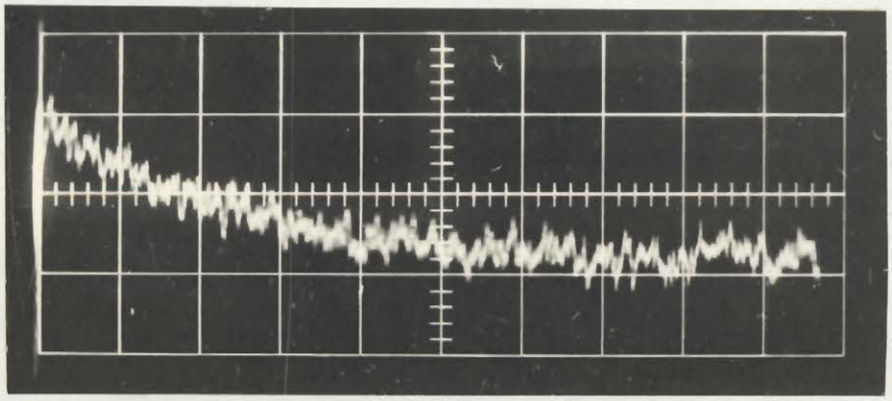
$[(\overline{P}) + (\overline{D})] = 2.7 \times 10^{-5}$ M



τ = 6 msec

5 msec/cm

$[(\overline{P}) + (\overline{D})] = 1.1 \times 10^{-5}$ M



τ = 10 msec

5 msec/cm

TABLE VII

Conditions Used in Relaxation Experiments for the System BSA : N-R' in Borate-KNO_3 Buffer (pH 8) at 25°C*

(P) total x 10^5 [M]	(D) total x 10^5 [M]	(D) bound x 10^5 [M]	$(\bar{P})$ x 10^5 [M]	$(\bar{D})$ x 10^5 [M]	$[(\bar{P}) + (\bar{D})]$ x 10^5 [M]	τ x 10^3 [sec]	$1/\tau$ x 10^{-1} [sec^{-1}]
0.86	1.20	0.45	0.41	0.75	1.15	19.7	5.1 ± 0.4
1.36	1.10	0.58	0.78	0.53	1.31	12.9	7.8 ± 0.8
2.62	2.20	1.08	1.54	1.12	2.66	11.4	8.8 ± 0.6
4.95	2.26	1.58	3.37	0.69	4.06	8.7	11.5 ± 1.6
7.20	2.20	1.72	5.50	0.47	5.98	6.0	16.7 ± 2.1

* Relaxation times of approximately the same magnitude were observed in "Tris-KNO_3 buffer. However, not many accurate measurements could be made in this buffer because of the extremely large changes in the protolytic equilibrium of this buffer-dye system.

$[(\bar{P}) + (\bar{D})]$ for 25°C, 30°C and 35°C, are shown in Fig. 20 and the corresponding rate constants, k_{-1} and k_1 at these temperatures are listed below.

Temperature °C	$k_{-1} \times 10^{-1}$ (sec^{-1})	$k_1 \times 10^{-6}$ ($M^{-1}sec^{-1}$)
25	3.5	2.1
30	7.0	3.2
35	13.6	3.7

The same overall mechanism was found to be valid for the reaction of NS-R' with BSA in phosphate buffer (pH 7.0, $\Gamma/2 = 0.1$) at 25°C (Fig. 21, Table VIII) and the corresponding rate constants were calculated as

$$k_{-1} = 2.5 \text{ sec}^{-1} \text{ and } k_1 = 3.5 \times 10^5 M^{-1}sec^{-1}$$

The equilibrium constants at 25°C obtained from the ratios of k_1/k_{-1} for the interaction of BSA with N-R' and NS-R' were $6.0 \times 10^4 M^{-1}$ and $1.5 \times 10^5 M^{-1}$, respectively. These values were of the same order of magnitude as the equilibrium constants calculated from static experiments, i.e. 1×10^5 and $5.5 \times 10^4 M^{-1}$ for the N-R' and NS-R' systems, respectively (c.f. page 93).

In view of the errors involved in the determinations of relaxation time, due to fluctuations in light intensity, and consequently in the evaluation of rate constants for the forward and reverse steps, the agreement between the values of the equilibrium constants, calculated from static and

FIGURE 20

Concentration dependence of $1/\tau$ for the reaction between N-R' and BSA in borate buffer (pH 8.0, $\Gamma/2 = 0.1$) at three different temperatures.

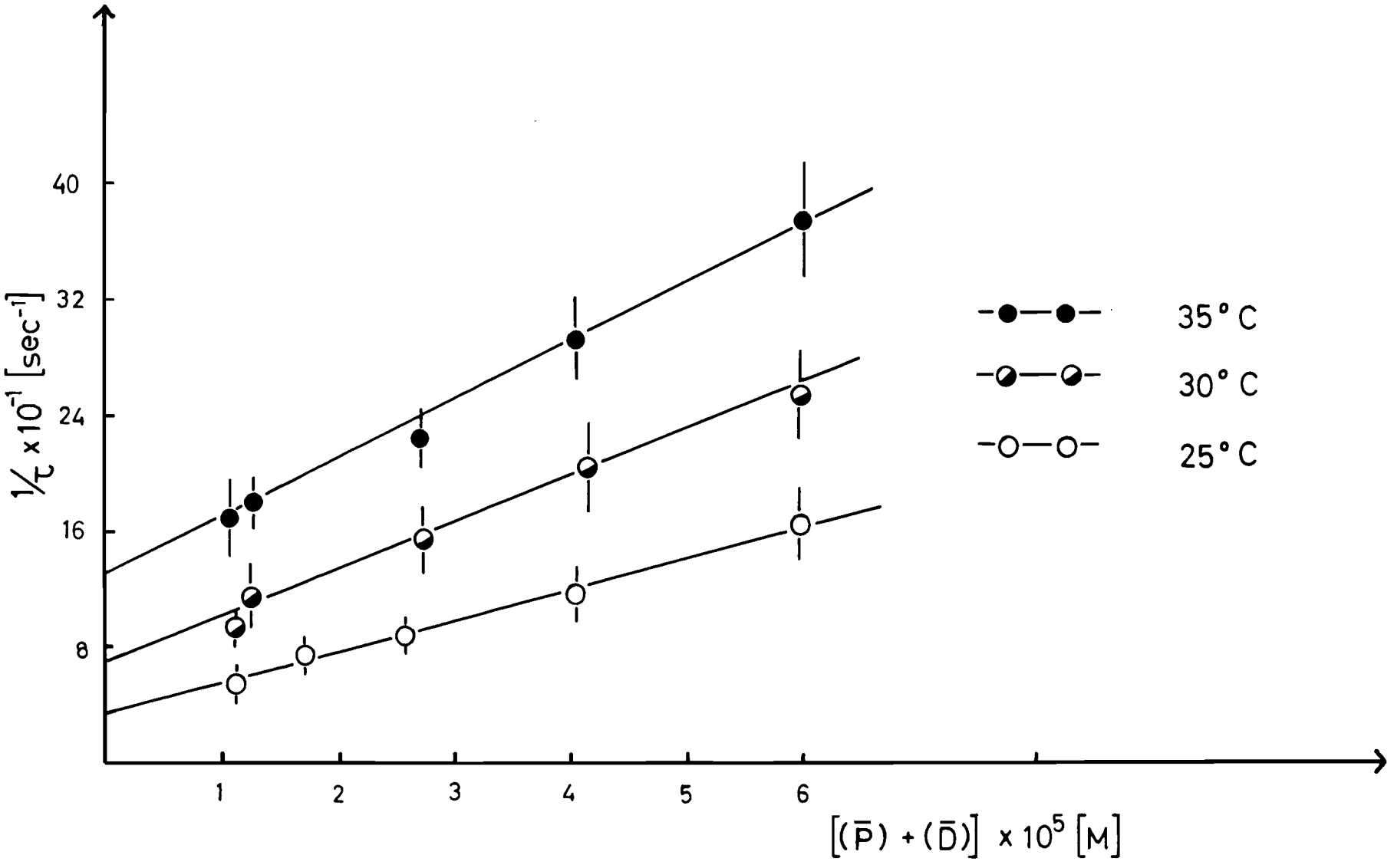

40
32
24
16
8
1/τ x10⁻¹ [sec⁻¹]
1
2
3
4
5
6
[(P̄) + (D̄)] x 10⁵ [M]
35° C
30° C
25° C

FIGURE 21

Concentration dependence of $1/\tau$ for the reaction between NS-R' and BSA in phosphate buffer (pH 7.0, $\Gamma/2 = 0.1$) at 25°C.

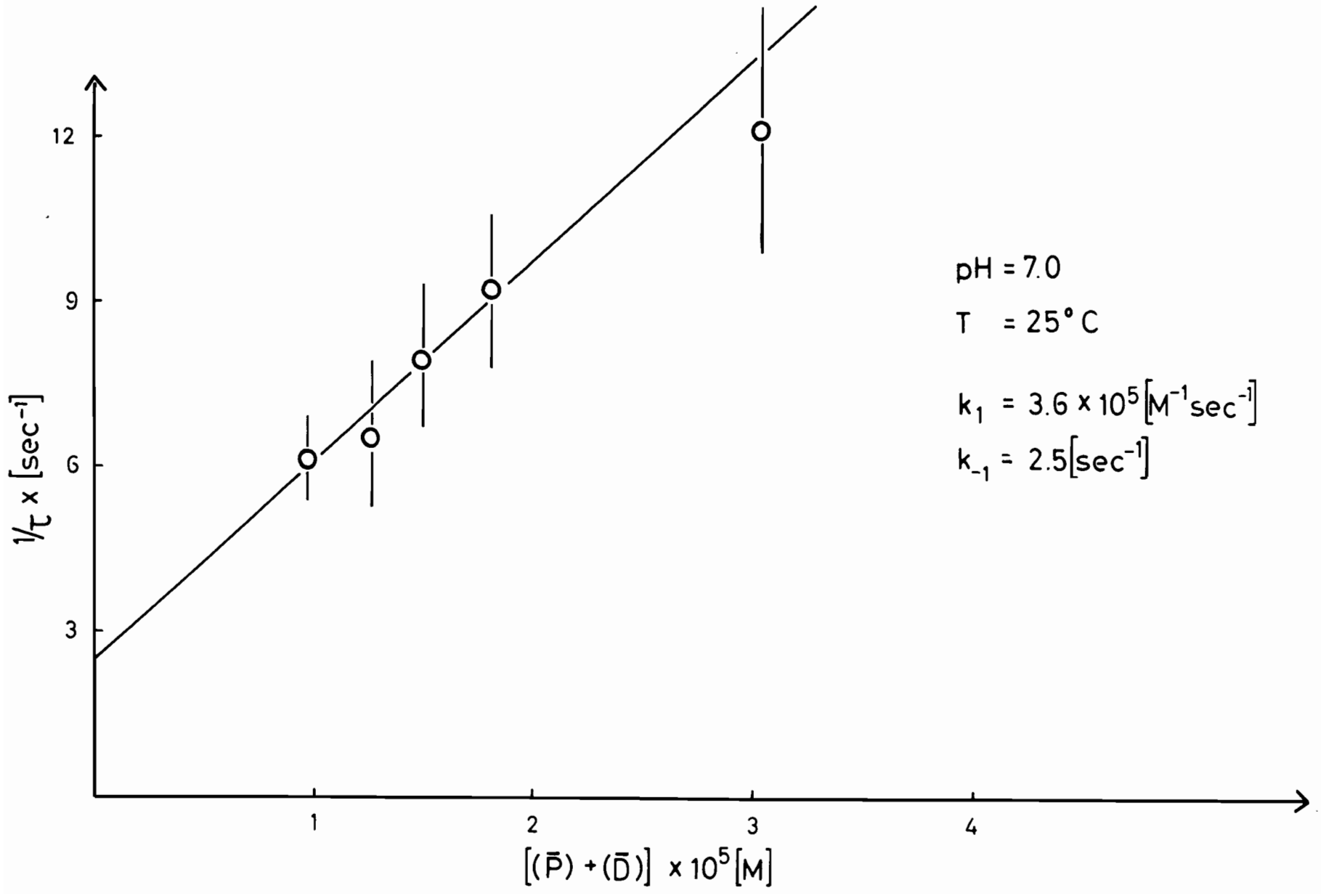

12
9
6
3
1/τ x [sec⁻¹]
1
2
3
4
[(P̄) + (D̄)] x 10⁵[M]
pH = 7.0
T = 25° C
k₁ = 3.6 x 10⁵[M⁻¹sec⁻¹]
k₋₁ = 2.5[sec⁻¹]

TABLE VIII

Conditions Used in Relaxation Experiments for the System BSA : NS-R'

(P) total $\times 10^5$ [M]	(D) total $\times 10^5$ [M]	(D) bound $\times 10^5$ [M]	$(\bar{P}) \times 10^5$ [M]	$(\bar{D}) \times 10^5$ [M]	$[(\bar{P}) + (\bar{D})] \times 10^5$ [M]	$\tau \times 10^1$ [sec]	$1/\tau$ [sec^{-1}]
0.27	1.0	0.14	0.12	0.86	0.98	1.59	6.3 ± 0.6
1.1	1.0	0.39	0.71	0.61	1.32	1.51	6.6 ± 1.8
1.8	1.0	0.64	1.16	0.36	1.52	1.27	7.9 ± 1.7
2.1	2.0	1.18	0.92	0.83	1.75	1.10	9.1 ± 2.0
3.3	3.0	1.58	1.67	1.42	3.09	0.82	12.2 ± 2.5

relaxation experiments, are considered satisfactory. These equilibrium constants are also of the same order of magnitude as those obtained for the binding of some alkyl sulphates (114) and of aromatic carboxylates (165) to BSA.

It ought to be also stated that in the static experiments (Figs. 17 and 18) the concentration of the dyes had to be varied within wide limits. In the case of N-R', some of these concentrations were outside the range where the dye obeyed Beer's Law. This effect was probably caused by micelle formation and might have introduced a serious error in the calculation of both the equilibrium constant and the number of binding sites. The latter error would be obviously carried over to the kinetic experiments in as much as the relaxation times were related to the total number of active sites on the albumin molecule.

In view of the heterogeneous population of binding sites with respect to their affinity for the dye, which resulted in a distribution of binding constants (e.g. as shown in Fig. 17), it is reasonable to expect that there should be also a statistical distribution of the corresponding rate constants, k_1 and k_{-1}, rather than single values as derived from relaxation experiments. From the equilibrium experiments with N-R' an average value for the binding constant was determined. However, on the basis of the calculated heterogeneity coefficient of 0.6, the actual equilibrium constants for the different binding sites would vary over a wide range of values, differing by as much as two orders

of magnitude (115). In consequence, at the lower concentrations of the dye, the contribution of the binding sites with the higher affinities will be more pronounced, and on increasing the concentration of the dye the binding sites having weaker affinities will come into play. No account was taken of this complication in plotting the concentration dependence of τ, from which the values of k_1 and k_{-1} were calculated.

Because of all these considerations it is obvious that the temperature dependence of the rate constants (Fig. 20, c.f. page 100) could not be obtained with a high degree of accuracy, which is required for the determination of activation energies. Moreover, the present studies were restricted (for technical reasons) to the narrow temperature range of 10°C, so that relatively small errors in the rate constants would lead to considerable uncertainities in the activation energies. Nevertheless, from the corresponding Arrhenius plots of log k versus $1/T$, the activation energies for the association and dissociation steps were estimated as approximately 10 kcal/mole and 20 kcal/mole, respectively. Since these estimates were calculated from the results obtained only for three temperatures, they should be regarded with some reservations.

It ought to be pointed out that the temperature-jump cells used in the present investigation were not provided with temperature-sensing elements, such as thermistors or micro-thermocouples for the accurate determination of the temperature

in the reaction zone of the cell. Therefore, the actual temperature, after the discharge of the condensor, could only be estimated by using equation (6) given on page 38.

In view of the great number of protolytic equilibria* involved in protein-dye-buffer systems, it is conceivable that the rates of albumin-dye interactions would be affected greatly by pH. Relaxation time measurements at three different pH values in borate buffer are shown in Fig. 22. From the slopes and the intercepts of the straight lines it can be seen that the dissociation rate constants increased and the association rate constants decreased with increasing pH. This could be explained partly by increased repulsion between the negatively charged reactants, which become more pronounced at higher pH values.

With regard to the magnitude of the association rate constants for BSA-dye interactions, which are of the order of $10^6 M^{-1} sec^{-1}$, it should be pointed out that these values compare favourably with the rate constants calculated for some enzyme-substrate reactions (166). Unfortunately, the rate constants obtained in this study cannot be compared with other rate constants for albumin-dye interactions, since no other data for such systems have been reported to date.

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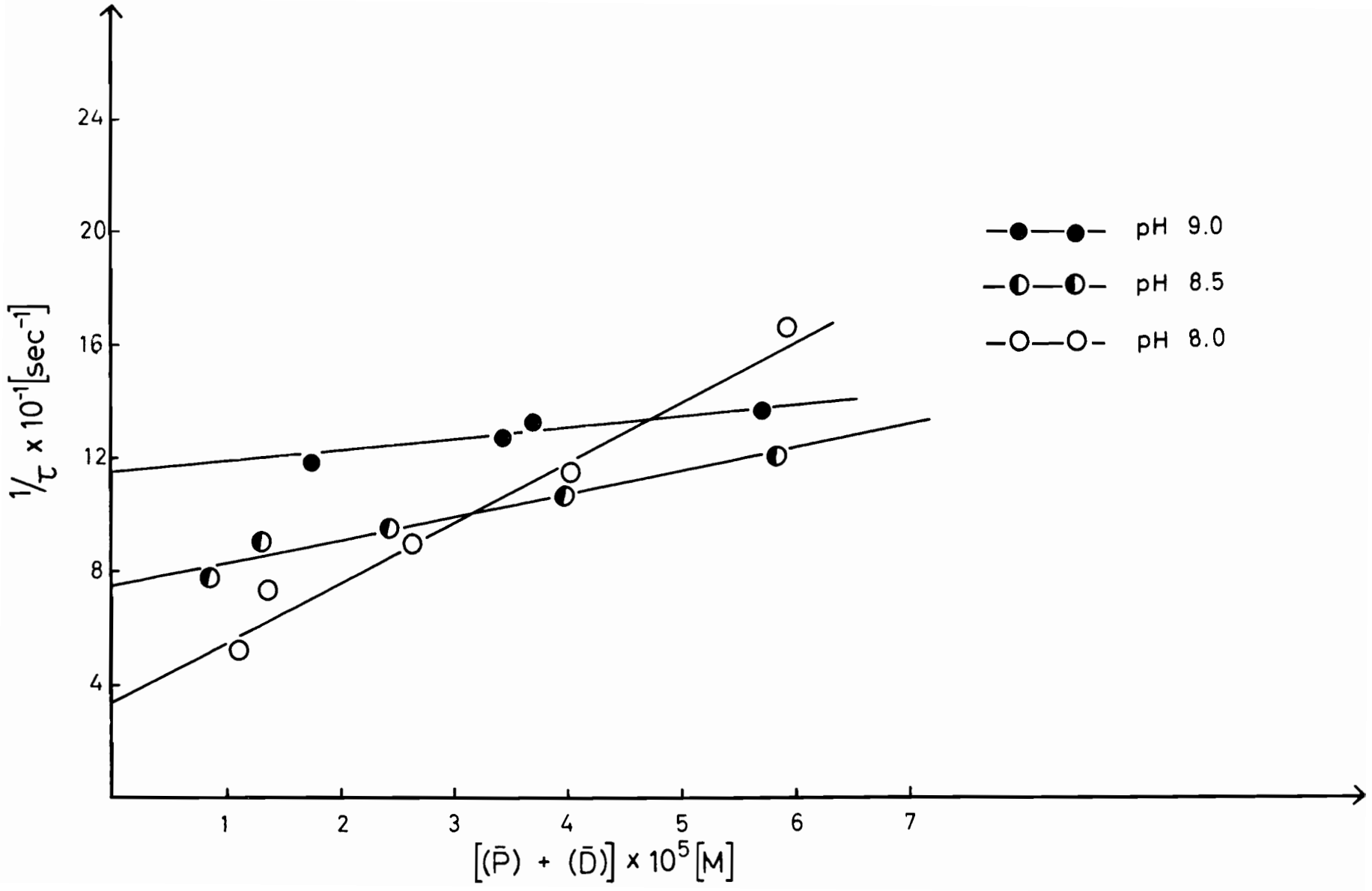
* Protolytic equilibria refer to reactions of H^+ and OH^- ions with their corresponding counterparts.

FIGURE 22

Concentration dependence of $1/\tau$ for the reaction between N-R' and BSA in borate-KNO_3 buffers of three different pH values.


24
20
16
12
8
4
1/τ x 10⁻¹[sec⁻¹]
1
2
3
4
5
6
7
[(P̄) + (D̄)] x 10⁵[M]
pH 9.0
pH 8.5
pH 8.0

(b) ANTIBODY-HAPTEN INTERACTIONS

Reactions of the haptens N-R' and NS-R' with anti-R antibodies, separated from several rabbit antisera, resulted in a shift of the spectra of both haptens to longer wavelengths, the shift being more pronounced the higher the concentrations of antibodies.

The change in the absorption spectrum of NS-R' due to the addition of antibodies in "Tris"-NaCl buffer (pH 7.5, $\Gamma/2 = 0.1$) is shown in Fig. 23. From the data plotted in Fig. 24 a value of 2.7×10^4 $M^{-1}cm^{-1}$ was calculated for ϵ_b at a wavelength of 610 mμ. On the other hand, addition of normal rabbit gamma-globulins to this hapten did not result in a spectral shift. There was, however, a small decrease in the extinction coefficient of the hapten at the wavelength corresponding to the maximum absorption of the free dye (Fig. 25). The shifts of the absorption spectrum of the dyes was, therefore, considered to be due to the specific binding of the hapten to anti-R antibodies.

Unfortunately, it proved impossible to measure relaxation times for the reaction between NS-R' and the antibodies in "Tris" buffer, because of the large temperature coefficient of the latter. This led to large changes in the protolytic equilibrium of the dye, which masked any effect due to the interaction of the antibody with the hapten. Phosphate buffer was not used either, since it was found to inhibit precipitation

FIGURE 23

The effect of anti-R antibodies on the absorption spectrum of a 4.8×10^{-6}M solution of NS-R' in "Tris"-NaCl buffer (pH 7.5, $\Gamma/2 = 0.1$).

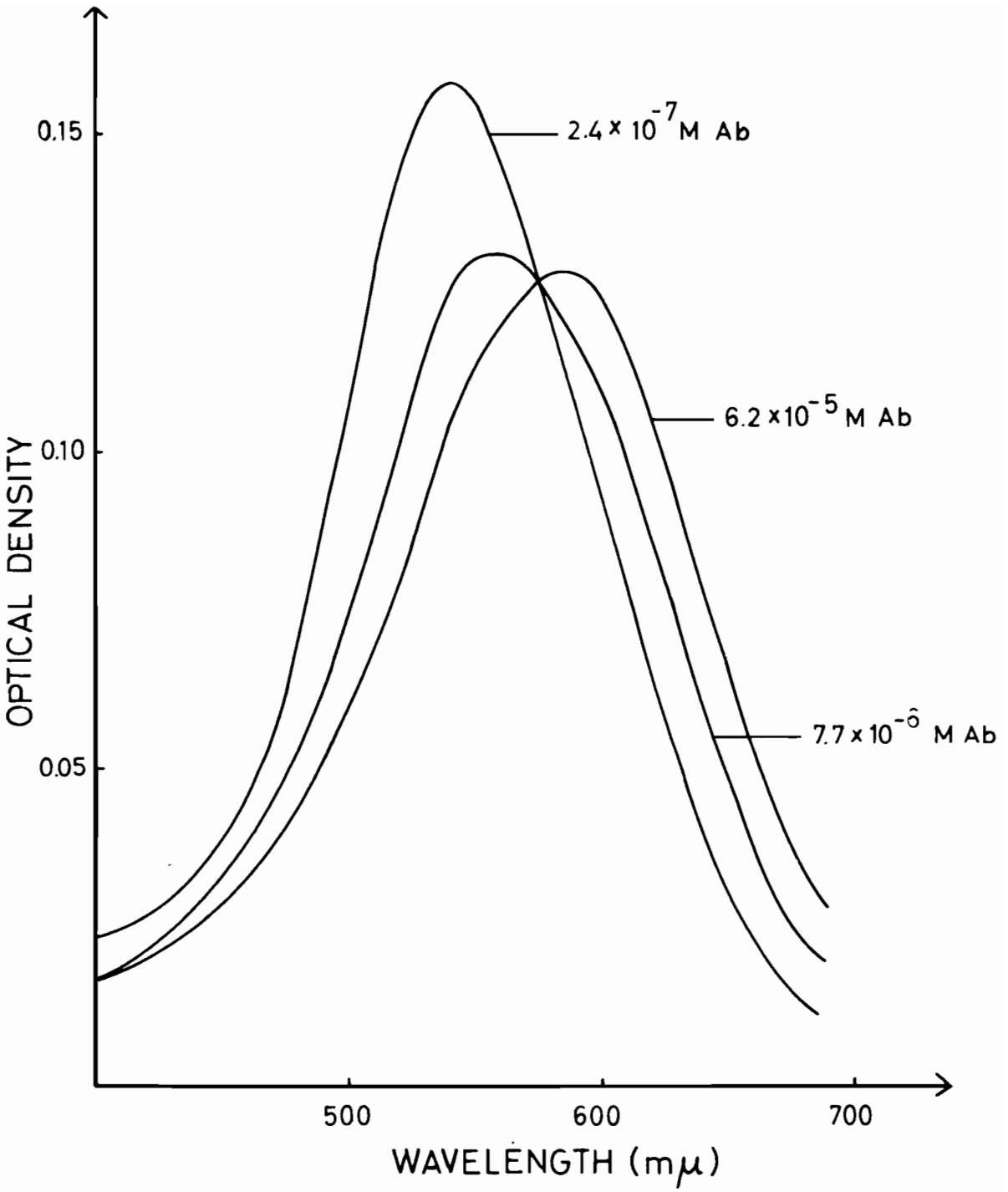

0.15
0.10
0.05
OPTICAL DENSITY
2.4 x 10^-7 M Ab
6.2 x 10^-5 M Ab
7.7 x 10^-6 M Ab
500
600
700
WAVELENGTH (mμ)

FIGURE 24

Determination of ϵ_b of NS-R', bound by anti-R antibodies, for two different dye concentrations in "Tris"-NaCl buffer (pH 7.5, $\Gamma/2 = 0.1$).

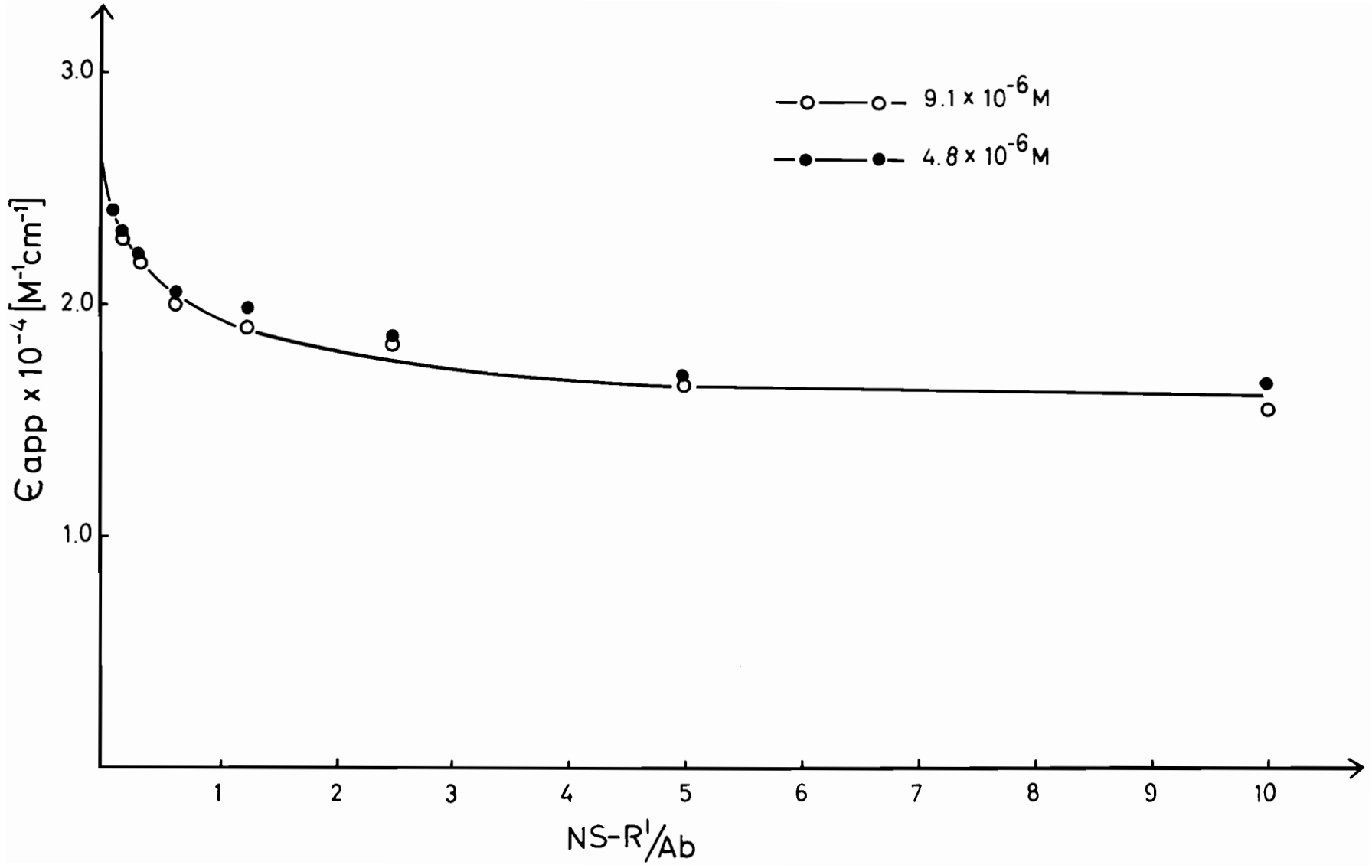
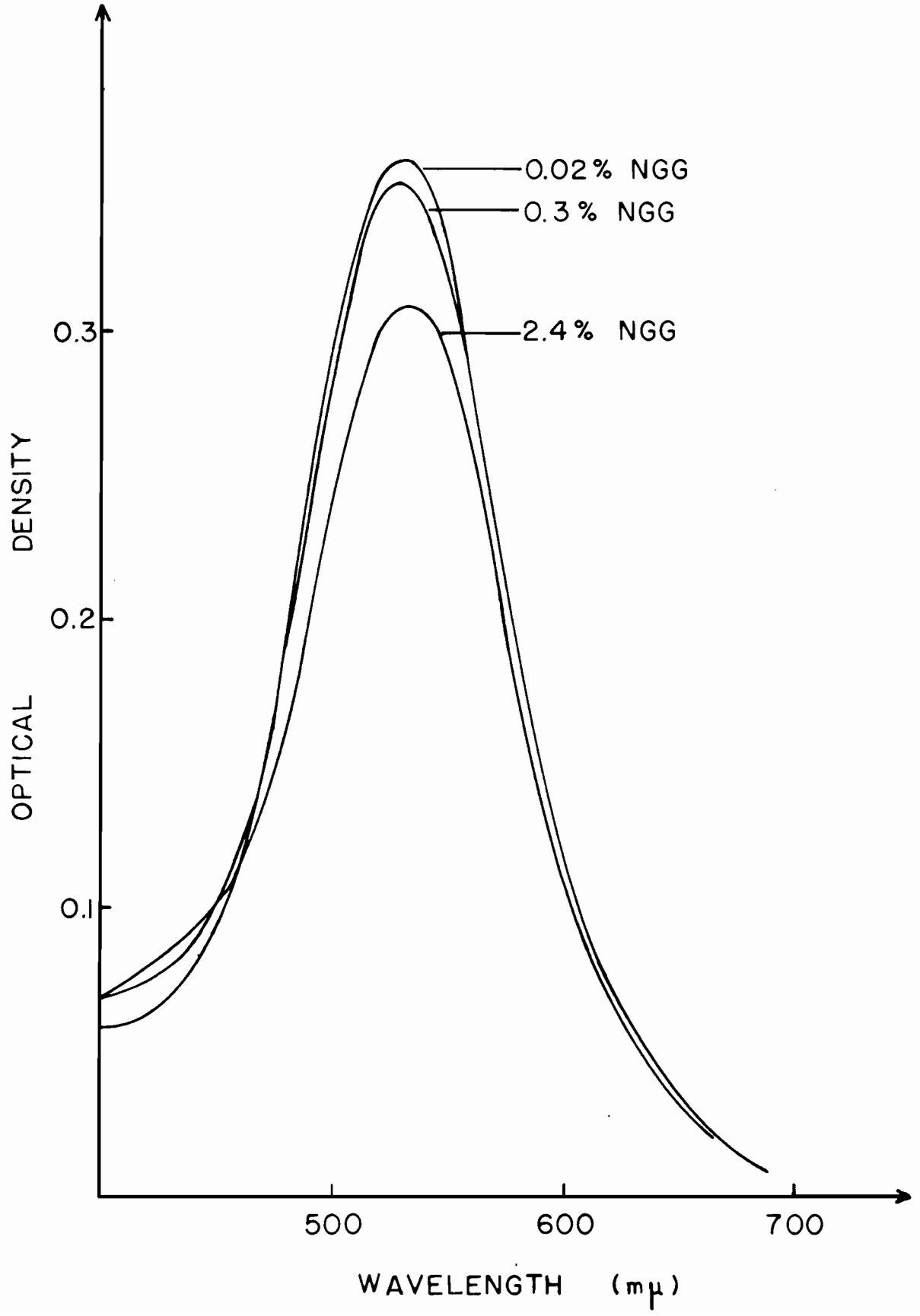

3.0
2.0
1.0
ϵ app x 10^{-4} [$M^{-1}cm^{-1}$]
—o—o— 9.1 x 10^{-6} M
—●—●— 4.8 x 10^{-6} M
1
2
3
4
5
6
7
8
9
10
NS-R'/Ab

FIGURE 25

The effect of normal rabbit gamma globulin on the absorption spectrum of a 9.1×10^{-6}M solution of NS-R' in "Tris"-NaCl buffer (pH 7.0, $\Gamma/2 = 0.1$).


0.02% NGG
0.3% NGG
2.4% NGG
0.3
0.2
0.1
OPTICAL DENSITY
500
600
700
WAVELENGTH (mμ)

of anti-R antibodies with homologous protein-hapten conjugates, as was also pointed out by other investigators (40).

In spite of the fact that the hapten N-R' was univalent, some precipitation was observed at certain antibody to hapten ratios. However, no precipitation was observed when either of the reactants was present in excess. This precipitation was most likely caused by the aggregation of the hapten molecules into micelles. For this reason no equilibrium studies were done with N-R'. Nevertheless, this system lent itself to the study of the kinetics of these reactions, since relaxation time measurements could be made with it in borate-NaCl buffer at pH 8.0, in a concentration range where no precipitation occurred (Table IX). The value for ϵ_f was calculated as 0.68 x 10^4 $M^{-1}cm^{-1}$ from optical density measurements with a solution of N-R' containing normal rabbit gamma-globulins. The value for the extinction coefficient of the bound hapten N-R' was not determined directly from measurements of ϵ_{app} for solutions of different ratios of hapten to antibodies because of the complications caused by the precipitation phenomenon referred to above. In consequence, the value of ϵ_b was assumed to be 2.8 x 10^4 $M^{-1}cm^{-1}$ by analogy with the value determined for the BSA : N-R' system. A relaxation effect for the antibody - N-R' system is illustrated in Fig. 26(a). By contrast, no relaxation effects were obtained with solutions containing the hapten N-R' in the presence of normal rabbit gamma-globulins; this is

TABLE IX

Conditions Used in Relaxation Experiments for the System Ab : N-R'
in Borate-NaCl Buffer (pH 8.0) at 25°C

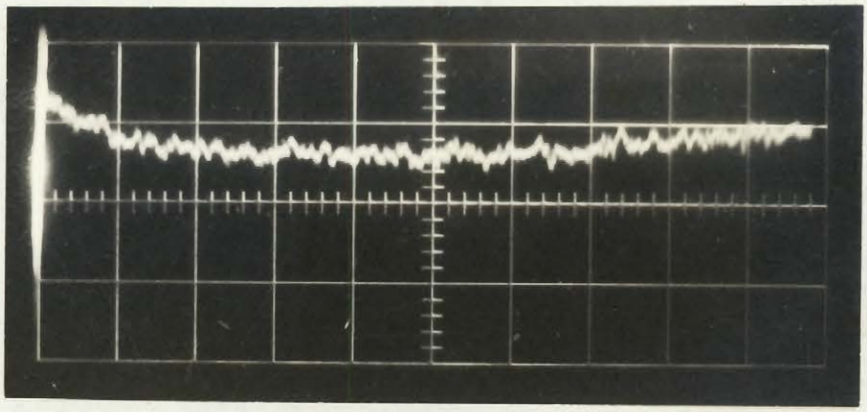
$(Ab)_{total}$ $\times 10^5$ [M]	$(H)_{total}$ $\times 10^5$ [M]	$(H)_{bound}$ $\times 10^5$ [M]	$(\overline{Ab})$ $\times 10^5$ [M]	$(\bar{H})$ $\times 10^5$ [M]	$[(\overline{Ab}) + (\bar{H})]$ $\times 10^5$ [M]	$\tau \times 10^3$ [sec]	$1/\tau \times 10^{-1}$ [sec^{-1}]
0.8	0.5	0.33	0.47	0.17	0.64	5.3	18.9 ± 1.4
0.8	1.0	0.45	0.35	0.55	0.90	3.8	26.3 ± 5.5
1.6	1.0	0.67	0.93	0.33	1.26	2.9	34.5 ± 2.0

FIGURE 26

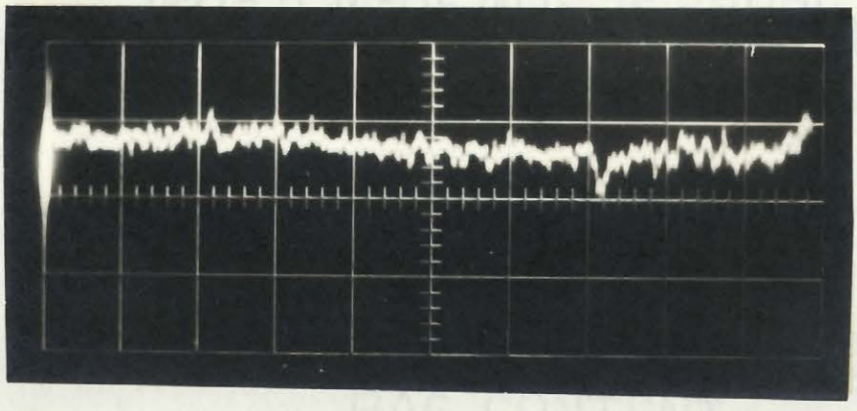
(a) Relaxation curve for the reaction of N-R' with anti-R antibodies.

(b) Oscillographic tracing for a solution containing normal rabbit gamma-globulin and N-R'.

(a)



(b)

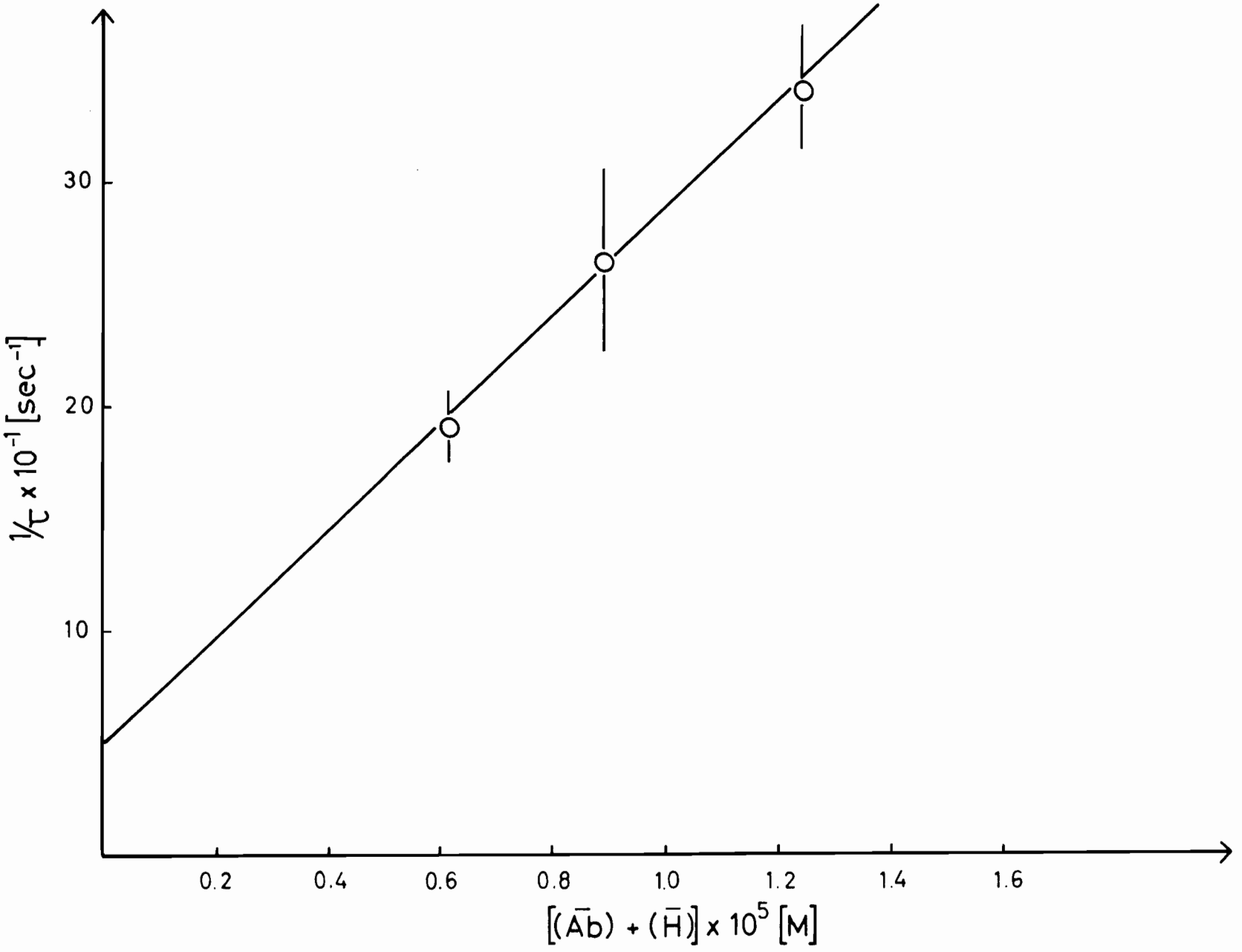


evident from the oscillographic tracing given in Fig. 26(b).

The data for three relaxation experiments obtained at different concentrations are listed in Table IX. From the concentration dependence of τ (Fig. 27), the rate constants k_1 and k_{-1} at 25°C were calculated as $\sim 2 \times 10^7$ $M^{-1}sec^{-1}$ and ~ 50 sec^{-1}, respectively. These values are within the range predicted by Schneider and Sehon (136) for the anti-R' antibody system. Moreover, the value calculated in the present investigation for the rate constant k_1 is also in agreement with the estimate obtained recently by Sturtevant et al (137) for the dinitrophenyl system. In addition, the binding constant of $\sim 4.0 \times 10^5$ M^{-1} calculated for this reaction from the ratio of k_1/k_{-1} agrees well with equilibrium constants computed for similar antibody-hapten reactions (103, 136). The rate constants for the association between N-R' and the specific antibody at 25°C was found to be larger than that for the reaction between N-R' and BSA (by a factor of about 10). This difference in rate constants may be accounted for by the larger repulsion between the negatively charged dye and BSA molecules at this pH, and by the fact that a greater extent of complementariness might be expected between the combining sites participating in the antibody-hapten systems.

FIGURE 27

Concentration dependence of $1/\tau$ for the reaction between N-R' and anti-R antibodies in borate-NaCl buffer (pH 8.0, $\Gamma/2 = 0.1$) at 25°C.


30
20
10
$1/\tau \times 10^{-1}$ [sec^{-1}]
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
[(Ab̄) + (H̄)] x 10^5 [M]

GENERAL DISCUSSION

Although the study of the kinetics of BSA-dye and albumin-hapten interactions is far from complete, it is felt that the purpose of this investigation has been achieved, in as much as it has been shown that the temperature-jump relaxation method lends itself to the investigation of these rapid reactions. Unfortunately, because of the large volumes of solution required with the present cells (50 cc capacity), only a limited number of experiments could be performed with the available supply of antibodies. Therefore, the usefulness of this method for the investigation of the speed of reactions between proteins and small molecules was established with the model system consisting of a BSA and dye haptens, N-R' and NS-R'. It has been shown that the rates of the reactions between albumin and the dye molecules, and between antibodies and dye-haptens could be followed by observing the changes in the optical properties of the dyes during the re-equilibration processes.

From the values derived for the rate constants of the association reactions between BSA and the dye molecules, N-R' and NS-R' (k_1 = 2.1 x 10^6 $M^{-1}sec^{-1}$ and 3.5 x 10^5 $M^{-1}sec^{-1}$, respectively), it can be calculated that the half-life times of these reactions at a concentration of about $10^{-5}M$ were

approximately 50 and 300 milliseconds, respectively*. Hence, it can be concluded that these protein-dye interactions could be also profitably studied by fast flow methods, as developed by Roughton (167) and Chance (168) for the investigation of the kinetics of some enzyme-substrate associations, since the deadtime of modern instruments is of the order of a few milliseconds (169). However, the major limitations of these techniques are incomplete mixing and cavitation of the solutions at the higher flow rates.

The rate constant for the reaction between anti-R antibodies and N-R' was computed as 2×10^7 $M^{-1}sec^{-1}$, which corresponds to a half-life time of about 5 milliseconds at the concentrations of antibodies used (10^{-5} M). It is not surprising, therefore, that Sturtevant et al (137) have recently found in a prelimary study that 60 percent of the reaction between their dye 2-(2,4-dinitrophenylazo)-1-naphthol-3, 6-disulphonic acid and the corresponding rabbit anti-DNP antibodies had occurred within 4 milliseconds, which was the deadtime of their stopped flow apparatus.

It is evident from the present investigation that the most useful dyes for the study of their interactions with proteins are compounds which are pH indicators, i.e. compounds

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* These half-life times were calculated with the aid of the expression: $t(1/2) = \frac{1}{ka}$, where k is the rate constant for the bimolecular reaction, and a is the concentration.

which exhibit significant changes in optical properties when their degree of ionization changes. It would appear that changes in the optical properties are brought about by structural changes of the dyes on combination with BSA or with antibodies. Similar conclusions can be also derived from the recent studies of the interaction of albumin with benzyl orange (170) and of a derivative of DNP with anti-DNP antibodies (137).

In the present study the absorption spectra of the dyes were shifted to longer wavelengths upon combination with BSA and anti-R antibodies. All spectrophotometric measurements were made at a wavelength, where the optical density was mainly due to the bound form of the dyes. A priori, measurements could have also been made at a wavelength were the free dye absorbed strongest. However, the latter procedure was not used since some irregularities were observed at these wavelengths. Thus, addition of small amounts of BSA to N-R' resulted in an increase in the optical density at the wavelength of maximum absorption of the free dye. Addition of larger amounts of BSA to solutions of this dye caused, however, a subsequent decrease in the optical density at these wavelengths. Similarly, the addition of normal gamma-globulins to a solution of NS-R' resulted in a decrease of the extinction coefficient at the wavelength of maximum absorption of the free dye; however, no spectral shift to longer wavelength was observed. The effect on the spectrum of the dyes due to the presence of normal gamma-globulins could,

of course, have been minimized by using specifically purified antibody solutions. This was not done since large quantities of antibodies were needed for the relaxation experiments and purified antibodies could only be obtained in yields of 60 percent as shown in Chapter V. It ought to be also pointed out that whilst the extinction coefficient of the free form of the dye was affected by the nature of the buffer ions (c.f. Figs. 12 and 13 for the absorption spectra of N-R' obtained in "Tris" and borate buffers at pH 8.0), there seemed to be no significant effect on the extinction coefficient of the bound form of the dye. No explanation can be offered at this time for the effects of protein and buffer ions at the wavelength of maximum absorption of the free dye, but it is believed that these effects may be partly due to changes in the dielectric properties of the solution, and were not the result of the specific interaction of the dyes with the proteins.

The fact that relaxation measurements could be made with the BSA-dye and antibody-hapten systems indicates that an overall change in enthalpy was associated with these reactions. It should be stressed, however, that the overall change in enthalpy may be due to several reactions involving in addition to the actual protein-dye interactions, protolytic reactions of the buffer ions, of the dyes and of the various ionizable groups of the proteins. Nevertheless, since the rate constants of most protolytic reactions are greater than 10^9 $M^{-1}sec^{-1}$,

(171) the relaxation effects observed are considered to represent the slower rate determining re-equilibration steps of the protein-interactions at the new equilibrium conditions after the temperature-jump. In effect, relaxation measurements with unbuffered solutions of the dyes, in absence of proteins, were much faster than those in the presence of BSA or anti-R antibodies.

On the basis of the concentration dependence of the relaxation time it has been postulated that all protein-dye interactions, which were studied, could be represented by the reaction

$$P + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PD$$

Moreover, from the temperature dependence of the two rate constants, k_1 and k_{-1}, it was deduced that the reaction of BSA with N-R' was exothermic to the extent of about 10 kcal/mole, i.e. $\Delta H = E_1 - E_{-1}$. However, as stated previously, in view of the narrow temperature range used in these experiments and in view of the inherent errors involved in the determination of the rate constants from relaxation times, this ΔH term ought to be considered with some reservation. The errors in the relaxation time measurements were brought about by two causes: (i) intensity fluctuations of the light source, which introduced irregularities into the exponential relaxation curves, and (ii) the

small relaxation effects obtained in some experiments. In spite of these errors, the fairly good agreement between the equilibrium constants, calculated for each of the BSA-dye systems from both kinetic and static experiments, and the reasonable value of 4×10^5 M^{-1} for the binding constant of anti-R antibodies with N-R', are considered to support the validity of the postulated mechanism and of the calculated values for the rate constants.

For most bimolecular diffusion-controlled reactions between relatively small molecules in solution, rate constants of the order of 10^9-10^{10} $M^{-1}sec^{-1}$ can be calculated (172). Alberty and Hammes (173, 174) adopted the existing theoretical expressions for diffusion-controlled bimolecular rate constants to enzyme-substrate reactions by assuming diffusion of the substrate into a hemispherical active site, located on a plane on the surface of the enzyme molecule. In terms of these assumptions the diffusion-controlled bimolecular rate constant for uncharged species was given by the expression

$$k = \frac{2\pi N}{1000} \cdot R_{1,2} D_{1,2}$$

In this equation N represents Avogadro's number, $R_{1,2}$ is the reaction radius and $D_{1,2}$ the sum of the diffusion coefficients of the interacting molecules. Using this equation for the antibody-hapten system, a rate constant of 1.5×10^9 $M^{-1}sec^{-1}$ was calculated. In this calculation the reaction radius was

taken as 4×10^{-8} cm, and the diffusion coefficients as 5×10^{-6} $cm^2 sec^{-1}$ and 4×10^{-7} $cm^2 sec^{-1}$ for the hapten and antibody molecules, respectively. This value for the rate constant is about 2 orders of magnitude larger than the experimental rate constant obtained in the present study. The above rate expression is, of course, an oversimplification, since interactions of coulombic forces were neglected. As postulated by Debye (172) and by Hammes and Alberty (174) an increased rate constant is to be expected if the reaction involves the interaction of oppositely charge species. However, if the reaction partners possess identical charges, the rate constant would be lower due to electrostatic repulsions.

According to the theory of absolute reaction rates (175), the rate constant for a bimolecular reaction in solution is given by the expression

$$k_1 = e \frac{kT}{h} \cdot e^{-E/RT} \cdot e^{\Delta S^{*}/R}$$

which enables one to calculate the entropy of activation provided the temperature coefficient of the rate constant is known. Regrettably, the temperature dependence of the rate constant for the antibody-hapten association was not determined because of the small supply of antibodies available at the time of these experiments. Therefore, an unambiguous explanation for the difference between the exponentially determined value of 2×10^7 $M^{-1} sec^{-1}$ for k_1 and the maximum theoretical value

of 1.5×10^9 $M^{-1}sec^{-1}$ (calculated on the assumption that the reaction was diffusion controlled and did not involve charged species) cannot be given, since this difference may represent the contributions of E and/or $\Delta S^{\ddagger}$.

The former term, i.e. the activation energy, might arise because of the electrostatic repulsions, at pH 7-8, between the negatively charged hapten and antibody molecules, or might represent the energy necessary for the reorganization of the water molecules in the combining sites. However, the problem is further complicated by the fact that, although the net charge of the antibody molecule is negative at pH 7-8, the charge of the antibody combining site, which is complementary to the negatively charged hapten, appears to be actually positive (40). In consequence, one might expect that the electrical fields due to the charges of the antibody combining site and of the hapten molecule would be reduced or annuled in the "transition complex" (175) and, hence, the water of solvation associated with the combining centres would be released (125). This effect would result in a positive term for the entropy of activation.

Although the activation energy of about 10 kcal/mole for the association reaction between BSA and the dye N-R' was not considered reliable, this value was used in conjunction with the rate constant calculated at 25°C for the evaluation of the entropy of activation. $\Delta S^{\ddagger}$ was thus computed as +1.4 e.u. The positive entropy could, as stated previously, be

interpreted in terms of a reorganization of the albumin molecule into more labile structure and/or the release of the water of solvation in the transition complex. Since in this system both reacting partners were negatively charged at the pH used, it is conceivable that the activation energy was mainly due to electrostatic repulsions. In principle, the contribution of electrostatic and non-electrostatic factors to the overall activation energy may be evaluated by studying the effects of pH, ionic strength and the alteration of the net charge of the protein and dye molecules, on the rate of reaction at different temperatures. The effect of pH was investigated in this study only at 25°C, and all one can deduce from these results is that the rate constant for the association step decreases and that the rate constant for the dissociation step increases with increasing pH, as would be expected for the interaction of two charged molecules.

From the equilibrium data for the BSA : N-R' system it was concluded that the binding sites of BSA were not identical with respect to their affinity for N-R'. The nature of these binding sites remains unknown and, considering the large configurational versatility of the albumin molecule (124), it is not too unlikely that these sites consist of chemically distinct groups having varying affinities for the dye molecule. The method used for the calculation of the heterogeneity constant does not take into consideration the nature of the binding sites and gives only a measure of the statistical distribution of

association constants. Because of similar considerations, the values of k_1 and k_{-1} derived in this investigation for the BSA : N-R' system must be regarded as overall rate constants for the association and dissociation processes involving the different binding sites. Such complications cannot be invoked for the BSA : NS-R' system, since it was shown that the BSA molecule possessed only one binding site for NS-R' and since no heterogeneity could be detected in this system. On the basis of the available evidence it has been generally accepted that an antibody preparation exhibits a certain degree of heterogeneity with respect to the binding affinities of the individual antibody molecules for the antigenically determinant group (115). However, the detailed chemical composition and structure of antibody sites are as yet not known. Therefore, it is impossible to state at this time whether the heterogeneity index calculated for an antibody preparation reflects a statistical distribution of the antibody combining sites with respect to the amino acid sequence or to the secondary and tertiary structures, which determine the configuration of the sites.

In conclusion, in view of the lack of more definitive information and in view of the limited scope of this study, the kinetic results obtained should be regarded as preliminary. Nevertheless, these results have established the range of values for the rate constants of protein-dye and antibody-hapten reactions, and have demonstrated the usefulness of the temperature-jump relaxation technique for the study of these reactions.

Although the values for the individual rate constants are subject to revision, it was gratifying to find that equilibrium constants calculated from kinetic data agreed fairly well with the binding constants derived from static experiments. It still remains to establish the validity of the spectrophotometric method used for the calculation of the extent of reaction in terms of a direct procedure such as equilibrium dialysis. This could be, in principle, achieved by using a greater variety of dyes and other semi-permeable membranes.

Moreover, it is hoped that the temperature-jump method could be improved by the use of light sources of higher intensity and greater stability, and by incorporation of temperature-sensing devices into the cells for the accurate measurement of temperature.

SUMMARY

1. Antigenically specific stroma-hapten conjugates were prepared by coupling of diazotized p-aminobenzene arsonic acid to bovine red cell stroma.
2. These conjugates were shown to remove antibodies specific to the phenyl arsonate group from rabbit antisera.
3. The absorbed antibodies could be eluted from the stroma-hapten conjugates by dissociation with an excess of the hapten or in acid medium at pH 3.2.
4. Using hapten elution, precipitating antibodies could be recovered with a yield of 60 percent and a purity of about 60 percent. It was suggested that the "impurities" were antibody gamma-globulins, combining sites of which had been blocked by hapten molecules, which could not be removed by dialysis.
5. Acid elution was shown to give yields of 40 percent and purities between 60 and 70 percent. From the nature of the precipitin curves obtained it was inferred that acid elution led to some alteration of the antibody molecule.
6. The two azo dyes, 4[4-(4'-azobenzeneazo) benzene arsonic acid]-1-naphthol (N-R') and 4[4-(4'-azobenzeneazo) benzene arsonic acid]-1-naphthol-2-sulphonic acid (NS-R') were prepared.
7. The absorption spectra of these dyes were shown to be shifted to longer wavelengths as a result of the interaction of the dyes with BSA or with antibodies specific to the phenyl arsonate group.

8. The extent of binding of these dyes by BSA and antibodies specific to the phenyl arsonate group was determined spectrophotometrically.
9. The temperature-jump relaxation technique was shown to be applicable to the study of protein-dye and antibody-hapten reactions.
10. With the aid of this technique the bimolecular rate constants of the combination of NS-R' and N-R' with BSA were determined to be of the order of 10^5 $M^{-1}sec^{-1}$ and 10^6 $M^{-1}sec^{-1}$, respectively.
11. The effect of temperature and pH on the rates of the reaction between N-R' and BSA were studied. The reaction was shown to be exothermic and the rate of association decreased with increasing pH.
12. The bimolecular rate constant for the reaction of N-R' with antibodies specific to the phenyl arsonate group was shown to be of the order of 10^7 $M^{-1}sec^{-1}$.

CLAIMS TO ORIGINALITY

1. Antibodies specific to the phenyl arsonate group were eluted from stroma-hapten conjugates using an excess of p-arsanilic acid. The yield of precipitable antibodies recovered was about 60 percent and their purity was also of the order of 60 percent.
2. It was shown that not all the eluting hapten could be removed by dialysis from the more strongly binding antibody sites; it was, therefore, suggested that the "impurities" were antibody gamma-globulins, the combining sites of which had been blocked by hapten molecules, tenaciously bound to some of the antibodies.
3. The absorption spectra of the dyes, 4[4-(4'-azobenzeneazo) benzene arsonic acid]-1-naphthol (N-R') and 4[4-(4'azobenzeneazo) benzene arsonic acid]-1-naphthol-2-sulphonic acid (NS-R') were shown to be shifted to longer wavelength as a result of the interaction with BSA or with antibodies specific to the phenyl arsonate group. The extent of binding of these dyes with protein molecules was determined spectrophotometrically.
4. The temperature-jump relaxation technique was shown to be applicable to the study of the kinetics of protein-dye and antibody-hapten reactions.

5. With the aid of this technique the bimolecular rate constants for the combination of NS-R' and N-R' with BSA were calculated to be of the order of 10^5 $M^{-1}sec^{-1}$ and 10^6 $M^{-1}sec^{-1}$, respectively. The corresponding rate constant for the reaction of N-R' with antibodies specific to the phenyl arsonate group was shown to be about 10^7 $M^{-1}sec^{-1}$.

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