STUDIES OF ANTIBODY-HAPTEN REACTIONS BY POLAROGRAPHY

by

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CHAPTER 1

THE NATURE AND PROPERTIES OF ANTIBODIES AND ANTIGENS

According to a historical survey of immunology by Grabar (1), the phenomenon of immunity was known to the ancient Greeks. A major breakthrough in the understanding of its physical basis was made in the latter part of the nineteenth century when Kraus (2) discovered that resistance to disease could be developed not only to intact bacteria, but also to their extracts or toxins. Von Behring and Kitasato (3) observed that this resistance was associated with the presence of some factor in immune serum, and subsequently 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.

The Properties of Antigens

All antigens have been shown to be macromoloecules, most of them of biological origin, such as blood cells, serum proteins, tissue extracts, conjugated proteins (4), polysaccharides (5), and lipids (6). More recently, it has been shown that also synthetic polymers such as polyvinylypryrrolidone (7) and polypeptides (8, 9, 10) possess

antigenicity. Sela et al. (11) have demonstrated that some synthetic polypeptides are antigenic when their molecular weight is as low as 4000.

In the early part of the twentieth century Landsteiner (9) showed that antigenicity could be conferred by well defined groups, such as benzoic and sulfanilic acid, provided they were coupled to proteins. These small molecules, which did not induce antibody formation when injected by themselves, were called haptens by Landsteiner. The reaction of haptens with their appropriate antibodies was found to exhibit extraordinary specificity. Thus, arsanilic acid-protein conjugates reacted with antibodies produced against this material, but not with antibodies to conjugates of proteins with benzoic or sulfanilic acid (9).

The Properties of Antibodies

In most cases antibody molecules are distinguishable from other serum proteins only by their ability to combine with the appropriate antigen or hapten. Their molecular weight is about 160,000 in most instances (12, 13), although antibodies with molecular weights of the order of 1,000,000 have been reported (14). On the basis of their electrophoretic behaviour they are classified usually as gamma-globulins (15, 13), although there is evidence from immunoelectrophoresis that some migrate with the velocity of alpha and beta-globulins (16).

The specificity of the reaction between antibodies and their appropriate haptens is postulated to result from complementariness in shape between the hapten and the sites on the antibody which combine with it (58). An analogous phenomenon is thought to be involved in the interaction of the combining sites of antibodies with the antigenically determinant groupings (that is, the groupings of the antigen molecule which combine with the antibody site) (18). The forces participating in these specific reactions are non-covalent and have been shown to be rather weak. These forces result from the cooperative interactions of electrostatic attraction between charges of opposite sign, Van der Waals forces, and hydrogen bonds (19, 20). More recently, the participation of hydrophobic bonds also has been invoked (21).

There are many experimental observations supporting the ideas outlined above, but only a few will be cited here. Thus, with regard to the role played by steric factors, it was found that antibodies to the p-azophenylarsonate group reacted with the benzenearsonate ion as well as with the benzenephosphonate ion, which is sterically related to the former. In contrast, the benzoate and sulfanilate ions, the charged groups of which have a steric arrangement differing from that of the two ions cited above, did not react

even though they are small enough to fit into the antibody site (22).

The participation of Van der Waals forces may be illustrated by the experiments of Pressman et al. (23) in which measurements were made of the relative affinity of antibodies to the p-azobenzoate ion for benzoic acid and its derivatives in which the para-hydrogen was replaced by different halogen atoms. It was found that the substituents which were more polarizable were more firmly bound, as would be expected if the halogen atom were bound as the result of London dispersion forces (24) or induction effects (25). Evidence for the participation of ionic forces was provided by experiments which showed that when the charged group of a hapten was replaced with one which was uncharged but similar in size and shape (e.g. the nitro group for the carboxyl group), the binding affinity decreased (26, 27, 28).

The Structure of Antibodies and of Their Combining Sites

Rabbit antibodies, the type used mainly in physicochemical investigations, appear in electron micrographs as cylindrical rods with a radius of 12-20Å and length of 200-240Å (29). From hydrodynamic measurements it was computed that the shape of the hydrated molecule in solution could be represented by a prolate ellipsoid with major and minor axes of about 340 and 38Å (30).

Rabbit antibodies have been shown to possess two sites (31-35) which seem to be located at the ends of the molecule (29, 36). The size of the site in antibodies to dextran has been estimated by Kabat (37) to be an area complementary to an extended chain of 6-7 glucose residues. The dimensions of the hexasaccharide molecule are 34 x 12 x 8Å. Karush (48) has obtained a similar value for two different antibody-hapten systems.

Very little is known about the composition of antibody sites. Several groups of workers have indicated that the combining site of some anti-hapten antibodies may contain the tyrosyl residue (38-40, 49, 50), and that it may contain a charge opposite to that of the hapten (87). There is also evidence that the antibody site may contain a charge opposite to that of the antigenically determinant grouping (88). Recently it has been shown that antibodies directed against different haptens have a small difference in their chemical composition (151). Thus, antibodies directed against the p- azophenylarsonate ion showed a significantly higher arginine and iso-leucine content, while the antibody directed against the p-azophenyltrimethylammonium ion showed a higher aspartic acid content. At the present time it is difficult to say whether or not these variations reflect differences in the structure of the antibody site or of other regions of the molecule.

Although little is known about the structure of the combining site, insight into some general features of the structure of the antibody molecules has been obtained in recent years. Porter (42) and Nisonoff and Woernly (43) have shown that papain cleaves rabbit antibody molecules to produce three fragments which could be separated by ion exchange chromatography on carboxymethylcellulose. Two of these fragments (I and II) had a molecular weight of about 50,000 and each contained a single antibody site. The other fragment (fraction III) had a molecular weight of about 80,000 and did not contain a site which could combine with antigen, but which may have contained the group responsible for fixing the antibody to tissues. Subsequently, Nisonoff et al. (44, 45) demonstrated that antibodies could be degraded by pepsin to yield a divalent fragment with a molecular weight of about 100,000. Upon treatment of this fragment with low concentrations of mercaptans, a single disulfide bond was split and two univalent antibody fragments were produced with properties similar to those produced by papain digestion. On the basis of these studies it was suggested that the antibody molecule consists of two nearly identical portions linked together by a disulfide bridge, which in turn is linked to a third fragment by covalent bonds (44).

Recently it has been shown that antibody molecules probably consist of two pairs of polypeptide chains with molecular weights of about 55,000 and 20,000 (46, 47, 63). Non-covalent forces play a role in maintaining the configuration of these chains in the intact molecule, since after reduction of five disulfide bonds (the intact molecule contains 22 (51)) the molecule retains its full immunological activity while the four chains may be separated by the action of urea, 1 molar acetic acid or 1 molar propionic acid.

Several attempts have been made to obtain information about the conformation of the polypeptide chains in intact gamma-globulins with the aid of optical rotatory dispersion measurements (130-134). Unfortunately, because the theoretical basis for the interpretation of such measurements has yet to be established unequivocally, certain assumptions must be made in interpreting the experimental data. As a result, depending on the assumptions made, different conclusions can be drawn. Thus, for example, Winkler and Doty (131) have argued that the molecule does not contain appreciable amounts of either a right handed α-helix or β structures, while Callaghan and Martin (130) have contended that both forms are present.

It is evident that the use of x-ray diffraction techniques for the determination of protein structure (52) would,

if applicable, perhaps provide the most unequivocal structural data. Although this method has been used successfully for myoglobin (53) and hemoglobin (54), the larger size of the antibodies and difficulties in obtaining crystalline derivatives would seem to preclude its use at this time.

Some In Vitro Manifestations of Antibody Reactions

There are many *in vitro* and *in vivo* manifestations of the reaction of antibodies with the appropriate hapten or antigen. Among the *in vivo* manifestations are phenomena such as hypersensitivity and anaphylactic shock, in which definite and distinctive physiological changes occur as the result of the antibody-antigen reaction (30). For studies of the physico-chemical factors underlying the mechanism of antibody-antigen reactions the *in vivo* manifestations are more relevant, and some of these are discussed below.

The Precipitin Reaction

Probably the most common *in vitro* manifestation of the combination of an antibody with its appropriate antigen is the formation of a precipitate when solutions of these materials are mixed. In general, the amount of precipitate which forms on the addition of increasing amounts of antigen to a constant amount of anti-serum is represented by a typical curve, known as the precipitin curve (55-57) (Fig. 1). It is evident from this curve that the amount

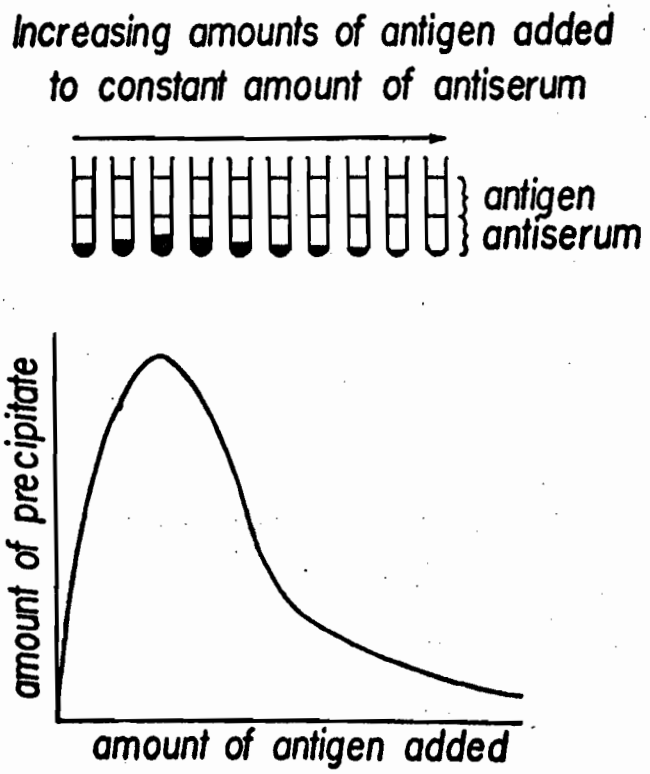


FIGURE 1

THE PRECIPITIN CURVE

of precipitate, consisting of both reactants, 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 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 framework theory (58). Accordingly, in the region of antibody excess the precipitate would consist of small aggregates composed primarily of antibody molecules crosslinked by the small number of antigen molecules: in the region of maximum precipitation, the antibody-antigen complexes would be crosslinked into larger and more compact aggregates consisting of an 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 crosslinking 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 substantiated 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 (59). Precipitating antibodies were shown to possess two combining sites as mentioned above. On the other hand, the univalent fragments I and II of rabbit antibodies prepared by enzymatic digestion, as mentioned on page 6, did not precipitate and actually inhibited the precipitin reaction (42), thus substantiating further the arguments of Pauling (58).

Goldberg (60) has attempted to develop a quantitative theory for the precipitin reaction on the basis of statistical arguments. The theory centers about an expression for the most probably distribution of antibody and antigen molecules among the aggregates of different size. Using this approach, Goldberg showed that at certain antibody-antigen ratios the system tends to change from one composed of small aggregates into one composed of relatively few, but exceedingly large aggregates, and this result was interpreted to mean that the precipitation would occur at these ratios.

Favorable agreement was obtained between the ratios predicted by the theory and those observed experimentally, but the course of the precipitin curve could not be predicted in detail. This may be due, at least in part, to the incorrectness of two assumptions made in deriving the expression for the most probable distribution: (i) the non-existence of cyclical structures in the aggregates, (ii) the equivalence of all antibody and antigen sites regardless of the size and shape of the complex in which they may be incorporated.

Ultracentrifugation and Electrophoresis

With the aid of the optical ultracentrifuge (61) or electrophoresis (62), the relative concentration of macromolecular components in solution which differ in size, shape or charge can be determined. Such differences exist in several instances among antibodies, antigens and their soluble complexes, so that the reaction of antibodies with antigens can be detected readily with these methods. Thus, in mixtures consisting of bovine serum albumin and its homologous rabbit antibodies, Singer and Campbell (35) found peaks in the schlieren patterns which could reasonably be attributed only to free antigen and soluble antibody-antigen complexes. Using the methods of ultracentrifugation

and electrophoresis, these authors were able to obtain the equilibrium constant for the reaction between antibodies and antigens and to calculate the corresponding changes in the standard free energy, enthalpy and entropy (35, 93). The values obtained at 0°C for bovine serum albumin and its homologous rabbit antibodies were $K = (2.5 \pm 0.5) \times 10^4$; $\Delta F° = 5.5 \pm 0.2$ kcal./mole; $\Delta H° = 0 \pm 2$ kcal./mole; $\Delta S° = 20 \pm 8$ e.u./mole.

The methods of ultracentrifugation and electrophoresis were applied also by Pepe and Singer (64) to study the reaction between a univalent antigen, prepared by coupling a hapten to the single available sulfhydryl group of bovine mercaptalbumin, with rabbit antibodies directed against the hapten. As predicted by the framework theory, precipitation did not occur, and from the ultracentrifuge patterns no evidence could be found for components sedimenting with a rate greater than that expected for the complex $Ab\text{-}Ag_2$.

Light Scattering

Since the amount of light scattered by a macromolecule in solution is proportional to its molecular weight (65), the intensity of scattered light increases when antibodies and antigens combine (66, 70). Several attempts have been made to use light-scattering measurements in investigations of the kinetics of these reactions (66-69). In all cases it was found that the change in intensity of scattered light was rapid within the first few minutes but then slowed down considerably. Although fairly precise data could be

obtained, evaluation of kinetic parameters for the initial reaction between the combining sites was difficult since the changes occurring during the first few minutes probably involve re-equilibration reactions among aggregates of different size (71), while changes determined during the later stages reflect primarily interactions among the aggregates themselves.

Only little use of light-scattering measurements has been made for equilibrium studies owing to difficulties in correlating the intensity of scattered light with the composition of the aggregates. However, in one case, these were overcome by the use of a system consisting of a divalent hapten containing two phenylarsonate groups and antibodies directed against the phenylarsonate residue (72). Light scattering measurements could be used to follow this reaction since in conformity with the lattice theory, only linear aggregates were formed.

Equilibrium Dialysis

The method of equilibrium dialysis has proved to be one of the most direct procedures for the study of the binding of small molecules by proteins (75) and has been widely applied to the evaluation of equilibrium constants of antibody-hapten reactions (31, 32, 73, 74). In this technique, two compartments are separated by a semipermeable membrane.

one containing the antibody (or protein) solution and the other the solution of the diffusible hapten (or of the small molecule). The concentration of hapten in the latter compartment is determined prior to, and after prolonged dialysis, and the amount of bound hapten is then calculated after equilibrium has been reached from the difference in these concentrations and from the volume of solution inthe two compartments. Control experiments are necessary to establish whether or not hapten is bound by proteins other than antibodies and/or by the dialysis membrane. If such "non-specific" binding occurs, appropriate corrections must be introduced. Experimental procedures and methods of treating equilibrium dialysis data are reviewed by Klotz (76) and Rosenberg and Klotz (75).

Quenching of Fluorescence

The quantum yield of the fluorescence of the tryptophyl residues of proteins may be decreased when a small molecule combines with the protein. A necessary condition for this to occur is that an absorption band of the small molecule overlap the emission band of the protein (77). Velick, Parker and Eisen (77) made use of these facts to develop a method for measuring the binding of haptens by antibodies. It is interesting to note that in one of the reactions studies by this method, i.e., the reaction between the hapten -N-2,4-dinitrophenyllysine and its homologous rabbit antibodies, the intrinsic equilibrium constant was

found to be 2 x 10^8 M^{-1}, which is one of the highest yet recorded for antibody-hapten reactions.

Recently, Day, Sturtevant and Singer (78, 79) adapted the fluorescence quenching method to study the kinetics of antibody-hapten reactions. Because of the rapidity of these reactions it was necessary to resort to a rapid-mixing, stopped-flow method in order to be able to follow them. For the combination of several dinitrophenyl haptens with rabbit antibodies directed against this group, the apparent second order rate constant was evaluated as $\sim 10^8$ l/mole./sec.

CHAPTER 2

PHYSICO-CHEMICAL STUDIES OF ANTIBODY REACTIONS

Intrinsic Equilibrium Constants and the Heterogeneity of Antibodies

Methods for evaluating the equilibrium constant for the interaction of antibodies with haptens from data obtained in equilibrium dialysis experiments have been summarized by Klotz (76). The relations used in these methods were derived from the law of mass action assuming the combining sites had the same intrinsic affinity for the hapten. One of these relations may be written as

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

Where r = the average number of hapten molecules bound per protein molecule, or the fraction of antibody sites occupied by hapten

$$= \frac{\text{number of bound hapten molecules}}{\text{number of antibody sites}}$$

n = the number of binding sites per molecule
c = the concentration of free hapten
K = the intrinsic equilibrium constant

The equilibrium constant K_i for the formation of the i^{th} complex is related to the intrinsic equilibrium constant by the equation

$$K_i = \frac{n - i + 1}{i} K \qquad (2)$$

According to equation(1), it should be possible to evaluate K from the slope of a plot of 1/r versus 1/c. However, for antibody-hapten reactions, a plot of this sort is not

linear and this has been attributed to the presence of antibodies with different affinities for the hapten (31, 32). Pauling et al. (81) postulated that the distribution of the equilibrium constants in such systems was Gaussian, and showed that on this basis the interaction between antibodies and haptens could be characterized by an average equilibrium constant and an appropriate index of heterogeneity. Using this postulate, Karush and Sonenberg (82) developed equations which permitted the evaluation of these parameters from data obtained in equilibrium dialysis experiments. The expression obtained for the fraction of antibody sites occupied as a function of the concentration of free hapten may be written as

$$\frac{r}{m} = 1 - \frac{1}{\pi} \int_{-\infty}^{\infty} \frac{e^{-\alpha^2}}{1 + K_o c e^{\alpha}} \, d\alpha \qquad (3)$$

$$\text{where } \alpha = \frac{1}{\sigma} \ln \frac{K}{K_o} \qquad (4)$$

In these relations K_o is the average equilibrium constant, σ is the index of heterogeneity, which is a measure of the range of values of K, and c is the concentration of unbound hapten.

The integral in equation(4) cannot be evaluated analytically but may be determined by graphical means. Karush and Sonenberg (82) have presented theoretical curves of $\frac{n}{r}$ versus $\frac{1}{K_o c}$ for a variety of σ's, and procedures for using them to evaluate K_o and σ from experimental data have been described

by Karush (83).

An alternate method of treating binding data in heterogeneous systems was employed by Nisonoff and Pressman (84) who made use of the equation.

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

which is essentially similar to the expression derived by Sips (85) for the adsorption of gases on solid surfaces. In this relation

b = the concentration of bound hapten
c = the concentration of free hapten
K_o = the average intrinsic equilibrium constant
Ab = concentration of antibody sites
a = index of heterogeneity

As shown by Sips, the distribution function satisfying equation(5) is almost Gaussian (85). According to this equation, if a = 1, the system is homogeneous, and the resulting relation is simply a form of the law of mass action. Under these conditions the slope of the plot of 1/b versus 1/c is equal to 1/KAb. For a heterogeneous system a plot of 1/b versus 1/c would yield a curve, the extent of curvature being dependant on the degree of heterogeneity. In this case, the index of heterogeneity, a, may be determined by finding the value of a which linearizes the plot of 1/b versus $(1/c)^a$.

The Thermodynamics of Antibody Reactions

Values of the order of -6 to -12 kcal. per mole have

been calculated for the change in the standard free energy, ΔF°, for the reaction between antibodies and the appropriate hapten or antigen (Table 1). It is evident, therefore, that the intermolecular forces participating in these reactions are rather weak. In Chapter 1 it is indicated that several types of non-covalent forces might be involved. In some instances the contribution of different forces for various parts of hapten molecules to the overall free energy change could be estimated. Thus, for the combination of haptens bearing charged carboxylate or tetramethylammonium groups with their specific antibodies, these groups were found to contribute -4.8 kcal./mole and -1.2 kcal./mole to ΔF°, respectively (26, 28). Rabbit antibodies to p-azopyridine showed a distinct specificity for the hydrated form of the heterocyclic nitrogen, and the contribution of the latter was about 2 kcal./mole greater than for a -CH= group in the same position (86).

The changes in entropy, ΔS°, and in enthalpy, ΔH°, have been determined for several antibody-hapten and antibody-antigen reactions, and the data are summarized in Table 1. In most cases these parameters were obtained from measurements of the average intrinsic equilibrium as a function of temperature followed by application of the relations

TABLE 1

THERMODYNAMIC CONSTANTS FOR ANTIBODY ANTIGEN REACTIONS

System	Material Reacting with antibody	ΔF° ($kcal M^{-1}$)	ΔH° ($kcal M^{-1}$)	ΔS° e.u.	Ref.
ε-N-dinitrophenyllysine: anti-DNP-antibodies	Univalent Hapten	-6.8	-1.6	17	90
ε-N-dinitrophenyllysine: anti-DNP-antibodies	Univalent Hapten	-11.3	-8.6	9	77
p-(p-dimethylaminobenzeneazo)-phenyl β-lactoside(Lac-dye): anti-Lac antibodies	Univalent Hapten	-7.25	-9.7	-8.8	31
D-phenyl-(p-(p-dimethylaminobenzeneazo)-benzoyl-amino)-acetate (D-I_p-dye): anti D-I_p-dye antibodies	Univalent Hapten	-7.24	-7.1	0.3	32
p-(tyrosineazo)-benzene sulfonic acid: anti-p-azobenzene sulfonate antibodies	Univalent Hapten	-8.94	-8.39	2	74
p-iodo benzoate: anti-azobenzoate antibodies	Univalent Hapten	-6.3	-4.1	7	91
Terephthalanide-p,p'-diarsonic acid: anti-azobenzene arsonate antibodies	Bivalent Hapten	7.4±2	0.8±2.6	22±9	92
Bovine serum albumin (BSA): anti-BSA antibodies	Protein	-5.5±0.2	0±2	20±8	63

TABLE 1 - CONT'D

System	Material Reacting with antibody	ΔF° ($kcal M^{-1}$)	ΔH° ($kcal M^{-1}$)	ΔS° e.u.	Ref.
Ovalbumin: anti-ovalbumin antibodies	Protein	-5.6 ± 0.2	0 ± 2	20 ± 8	93
Ribonuclease: anti-ribonuclease antibodies	Protein	-4.5 ± 0.2			94
Human serum albumin (HSA): anti-HSA antibodies	Protein	7.5-8.0	3.66	13.1-14.8	95
BSA-azobenzene arsonate conjugate: anti-azobenzene arsonate antibodies	Multivalent hapten-protein conjugate	-4.8 ± 0.2	0 ± 1	18 ± 4	96
BSA-phenylarsonate conjugate: anti-azobenzenearsonate antibodies	Univalent hapten-protein conjugate	-5.0			64

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

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

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

These relations may be employed only for reversible reactions, and the reversibility of antibody reactions has been demonstrated by several investigators (30-32, 89) and also in the present study as shown on page 122. In computing the value of K_o, all activity coefficients were taken as being equal to unity on the basis of the assumption that since the reactions were studied under conditions where the concentration of reagents was low (about 10^{-6} molar) the laws of ideal solutions would apply.

Since the combination of an antibody molecule with a hapten or an antigen is an association process, it would be expected that because of losses in rotational and translational degrees of freedom, ΔS° should be negative. However, as can be seen from Table 1, ΔS° is usually positive with the single exception of the reaction of the hpaten p-(p-dimethylaminobenzeneazo)phenyl β-lactoside and its homologous antibodies. Generally, ΔS° is larger for the reaction of antibodies with bivalent haptens or with macromolecular antigens than for the reaction with univalent haptens.

To account for the positive ΔS°, it has been proposed that the association process is accompanied by effects which

increase the degree of disorder of the overall system. Klotz (97) and Doty and Meyers (98) suggested that this increase is due to release of the water of hydration from the combining sites as a result of the neutralization of ionic charges present in the corresponding sites on the antibody and hapten molecules. More recently it has been pointed out that the association of non-polar residues in aqueous media, a process referred to as hydrophobic bonding, may also be associated with a positive $\Delta S°$, and Kauzmann (21) has suggested this type of bonding may be involved also in antibody reactions. The positive entropy change associated with this type of bonding is attributed to an increase in the degrees of freedom of water molecules that had been in the neighborhood of the non-polar groups before association (21, 99).

On the basis of the data available at the present time, it is difficult to determine the contribution of the factors discussed above to the positive change in entropy. Moreover, the origin of $\Delta H°$ has yet to be explained satisfactorily, and the detailed structure of the antibody site is not known. Therefore, a unified picture of antibody reactions at a molecular level cannot be presented at this time.

The Kinetics of Antibody Reactions

Information about the mechanism of chemical reactions

may be obtained from kinetic studies. However, at the time this study was initiated, and during its course, little was known about the kinetics of antibody-antigen and antibody-hapten reactions*. Several attempts had been made to measure the speed of the reaction of polyvalent antigens with divalent antibodies (66-69) by following the changes in the intensity of scattered light as a function of time. Such systems involve complex overall reactions leading to the formation of aggregates of different size. Therefore, it is conceivable that the initial reactions between the specific combining sites were obscured by re-equilibration steps resulting in some statistical redistribution of these aggregates with respect to size (71). In kinetic studies of the inhibition of luciferase (100) and the neutralization of bacteriophage (101) by the corresponding antibodies, the rate constant for the combination of the antibody and antigen was found to be of the order of $10^5 M^{-1} sec^{-1}$. However, the actual antibody-antigen reaction was not measured directly since biological methods were used to measure the concentration of either the antibody or antigen. For the combination of I^{131} labeled insulin with the corresponding antibodies, Berson and Yalow (102)

* For personal reasons this thesis was not submitted until August 1963, although the experimental work had been completed some time earlier and the results dealing with the kinetics of hapten-antibody reactions had been reported previously in a number of publications from this laboratory (105, 106, 71). It is to be noted that the publication of these results precedes the publication of the kinetic data obtained for antibody-hapten reactions by Sturtevant, Wofsy and Singer (104), Day, Sturtevant and Singer (78, 79) and Froese, Sehon and Eigen (103).

found the rate constant for the association reaction, k_f, to be of the order of $10^9 M^{-1} sec^{-1}$. The rate constant for the dissociation reaction, k_r, was computed to be 1 sec^{-1} on the basis of the measured equilibrium constant for the reaction, K, and the relation $K = \frac{k_f}{k_r}$. This reaction was followed by measuring the concentration of free I^{131}-insulin, after separating it from the other components by paper electrophoresis. Concentrations of I^{131}-insulin as low as $10^{-9} M$ were used, which slowed the reaction down to the extent that it could be followed by the method employed.

The kinetics of antibody-hapten reactions were investigated recently by Froese, Sehon and Eigen (103), Sturtevant, Wofsy and Singer (104) and by Day, Sturtevant and Singer (78, 79). In view of the exceedingly high rates of these reactions, special techniques were used for their measurement. The former group of workers used the temperature-jump relaxation method (107) to determine the rate of the combination of 1-napthol-4[-4-(4'azobenzeneazo)phenylarsonic acid] with rabbit antibodies directed against the phenylarsonate ion and found k_f to be 2×10^7 $M^{-1} sec^{-1}$ and k_r to be 50 sec^{-1}. A spectrophotometric method which depended upon the shift in the absorption spectrum of the hapten which occurred when it was bound by the antibody site was used to follow the reaction. The latter group of workers employed a stopped-flow method in conjunction with the fluorescence quenching tech-

nique to determine the rate of combination of antibodies against the 2,4-dinitrophenyl residue (DNP) with DNP-lysine, DNP-caproic acid and DNP-azo-1-3,6,disulfonic acid and found k_f to be 10^7 -10^8 $M^{-1}sec^{-1}$ and k_r to be $\simeq 1$ sec^{-1}.

CHAPTER 3

THE PRINCIPLES OF OSCILLOGRAPHIC POLAROGRAPHY

The polarographic method of analysis is based on the proportionality between the concentration of a compound and the current yielded when it is reduced (or oxidized) at the surface of an electrode (108). In its essentials, the technique consists of electrolysing a solution of the compound of interest in a cell provided with a dropping mercury electrode (referred to hereafter as DME) and a non-polarizable electrode. A potential is applied across the two electrodes and is varied, usually over a range of two volts, while the current flowing through the cell is measured. By appropriate analysis of the resulting current-voltage curves (polarograms), the magnitude of the reduction (faradaic) current may be determined (108).

In many of the conventional polarographic procedures, referred to hereafter simply as conventional techniques, the potential applied to the DME must be varied at such a low rate that it takes about ten minutes to traverse the voltage range of interest (108). By contrast, following the work of Matheson and Nichols (109), methods have been devised using vacuum tube circuits with which the voltage is scanned so rapidly that a complete polarogram is obtained within a fraction of a second, and within the lifetime of a single mercury drop. Since the current-voltage curves obtained with these techniques are presented on the screen of a cathode ray tube (CRT) instruments in which they are employed are called oscillographic or cathode ray polarographs.

FIGURE 2

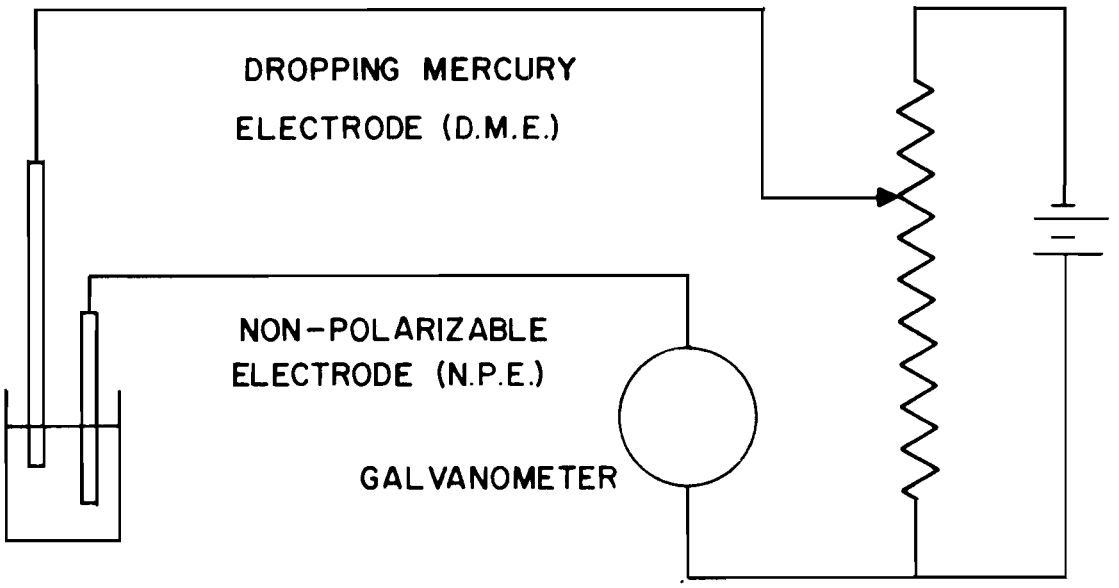
(Opposite side)

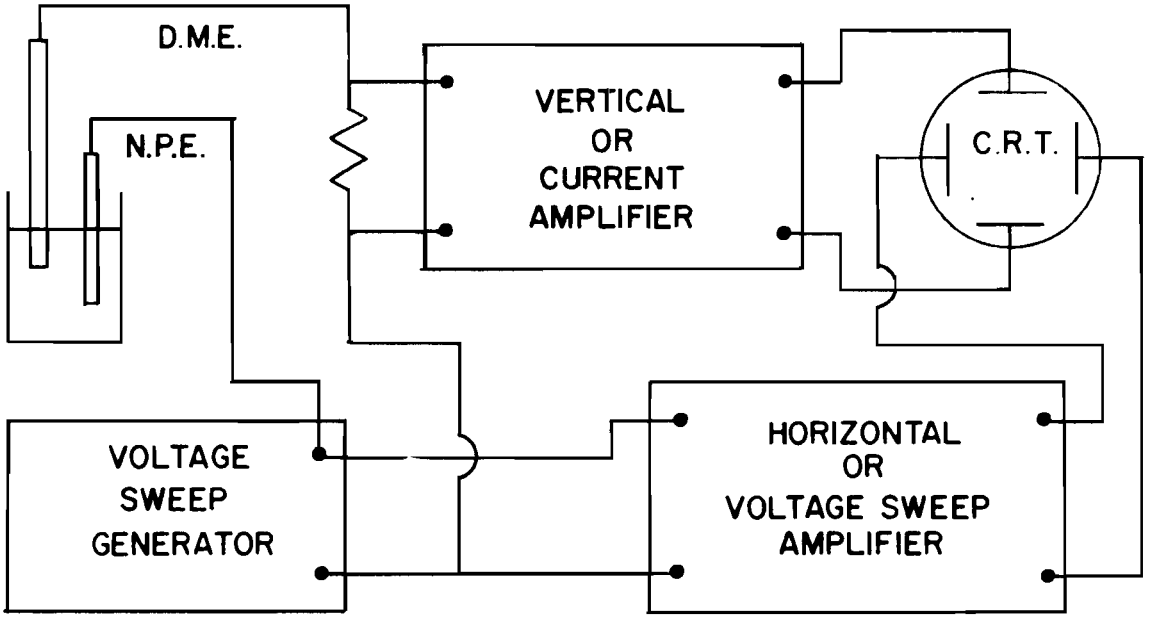
FIGURE 2

SCHEMATIC DIAGRAM OF CIRCUITS USED IN CONVENTIONAL AND OSCILLOGRAPHIC POLAROGRAPHS

Upper Diagram - conventional polarograph

Lower Diagram - oscillographic polarograph





The voltage applied to the electrodes during oscillographic polarography may vary in a linear or harmonic manner with respect to time (110). The present discussion is limited to the so-called single sweep instruments in which a single, linear sweep is applied only once during the lifetime of each mercury drop. The drop and sweep times under consideration are those varying from 2-7 seconds and 0.01 to 1 seconds, respectively.

Schematic block diagrams depicting the essential components of conventional and oscillographic polarographs are shown in Fig. 2. In conventional polarographs, the potential is applied directly to the electrodes by means of a potentiometer while the current is measured with a galvanometer (108). On the other hand, in oscillographic polarographs a voltage sweep generator is used to apply the polarizing voltage to a series combination of the polarographic cell and a resistor, while the current flowing through the cell is measured by the voltage drop it causes across the series resistor. This voltage drop is then amplified and fed to the vertical plates of a CRT, while the sweep voltage is also amplified and applied to the horizontal plates (110).

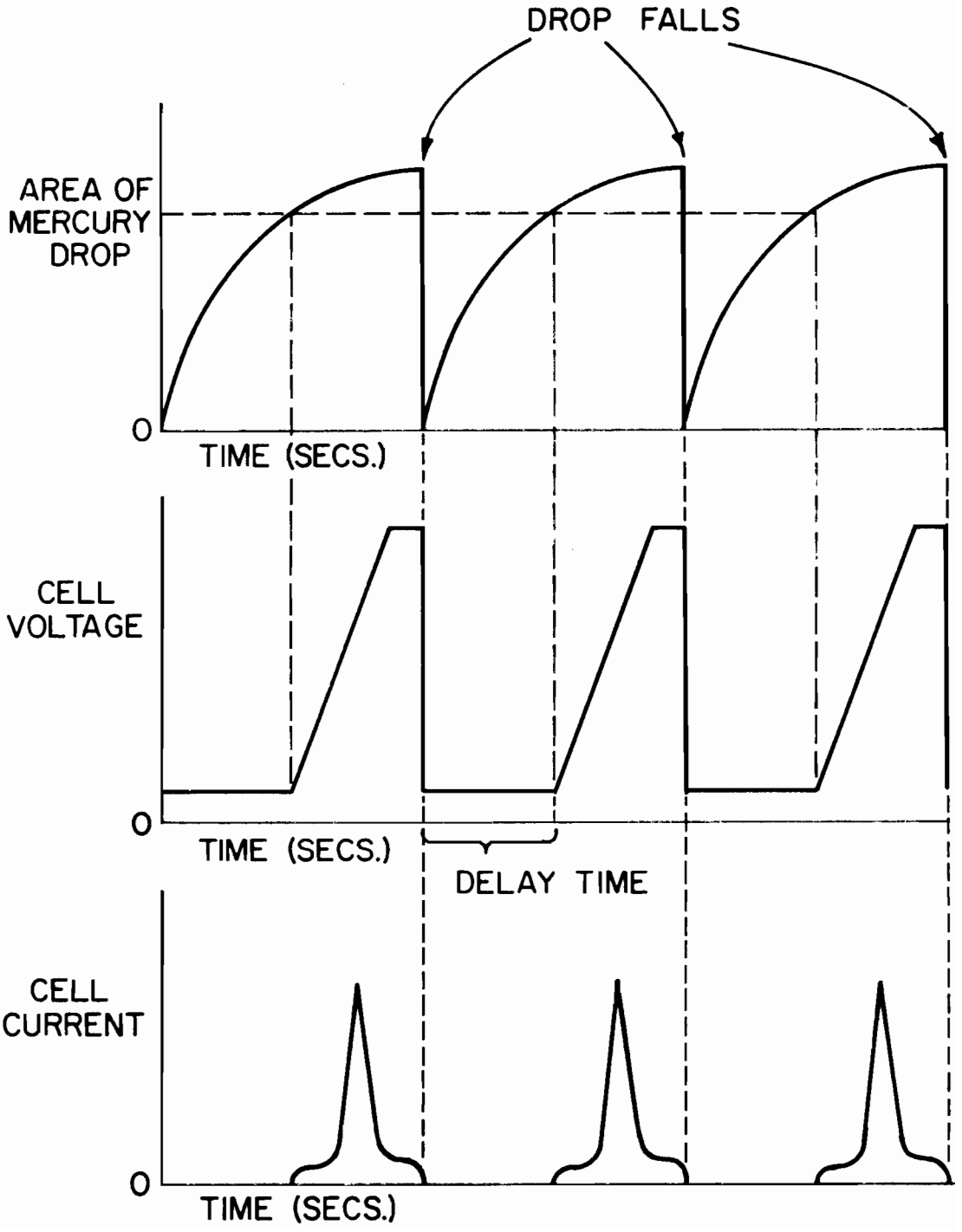
The current flowing through the cell under a given set of conditions is proportional to the surface area of the mercury drop (110). Therefore, to obtain reproducible results with oscillographic techniques, the start of the voltage sweep must be synchronized with the size of the

FIGURE 3

(Opposite side)

FIGURE 3

SEQUENCE OF EVENTS OCCURING DURING THE OPERATION OF AN OSCILLOGRAPHIC POLAROGRAPH



drop in such a manner that the sweep starts only when the drop has attained a certain size. Synchronisation may be accomplished by several methods (109, 112-116) some of which employ vacuum tube circuits. Of particular interest is the method in which an electro-mechnical device is employed to dislodge the mercury drops from the electrode by tapping the latter at definite intervals of time (115, 116). This device is used to simultaneously (i) dislodge a drop from the DME, and (ii) apply the voltage sweep when the drop has reached a certain size.

When the proper synchronisation is achieved, the oscillographic polarograph operates in a cyclic manner which is represented diagrammatically in Fig. 3. The sequence of events is as follows: (i) after a mercury drop is dislodged, a constant voltage is applied to the cell (usually 0 volts) until the new drop grows to the desired size, (ii) at this time the voltage increases linearly to a predetermined maximum and is maintained at this value until the drop falls or is knocked off, (iii) following detachment of the drop, the applied potential is decreased to the value it had originally and the cycle is repeated.

Conventional and Oscillographic Polarograms

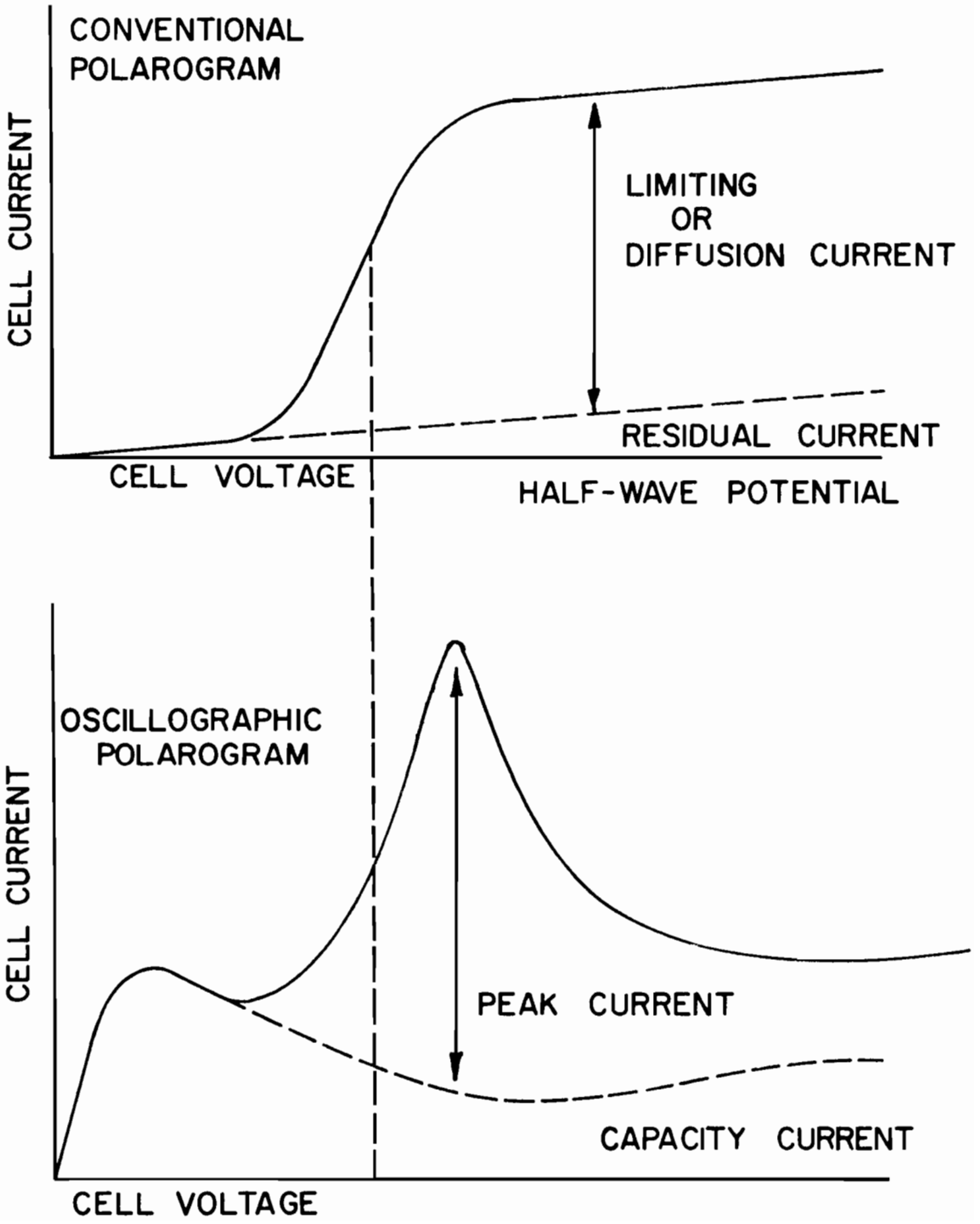
The current-voltage curves obtained with conventional and oscillographic polarographs for the reduction of a depolariser show distinct differences, the most obvious being that the faradaic current reaches a limiting or plateau value in the case of the former, and a peak value in the case of the latter. These differences, illustrated diagrammatically

FIGURE 4

(Opposite side)

FIGURE 4

CONVENTIONAL AND OSCILLOGRAPHIC POLAROGRAMS



in Fig. 4, are the direct result of the experimental techniques employed.

A limiting current is obtained with conventional techniques (108) since the polarizing voltage is applied continuously and the solution is not stirred during measurement. As a result, the amount of reducible material in the immediate vicinity of the electrode surface is rapidly exhausted. A fresh supply of depolariser is brought to the electrode by diffusion, and, within a very short time, a steady state is reached if the amount of depolariser available for reduction is supplied solely by diffusion. Under the conditions usually employed for polarographic experiments the rate of diffusion is independent of potential, with the result that the reduction current becomes limited only by diffusion.

Whenever measurements are made with a conventional polarograph in the absence of a depolariser, a small current is observed to flow, which, to a first approximation, is a linear function of the applied potential (residual current). This current is due to the reduction of minute amounts of impurities in the supporting electrolyte, and is also due to the current which must flow in order to charge up each new mercury drop to the required potential (108). This latter component is referred to as the capacity current. The residual current sometimes contributes significantly to the overall flow of electricity in the cell even in the presence of depolariser, and therefore, must be taken into account

in precise determinations of the plateau or limiting current.

The origin of the peak current in oscillographic polarograms is explained qualitatively in the following way (118). Prior to the application of the voltage sweep, the concentration of depolariser in the immediate vicinity of the mercury drop is higher than that which would obtain if a potential sufficiently negative for reduction were applied continuously to the cell. When the potential sweep occurs and the potential at which reduction can occur is reached, current begins to flow, and this current increases as the potential increases. However, as reduction occurs the concentration of depolariser in the immediate vicinity of the surface of the mercury drop begins to decrease, and perforce, the current drops. Thus, the current rises to a maximum and then decreases. During the voltage sweep, diffusion of depolariser towards the electrode surface tends to compensate for any depletion caused by reduction, but this compensating effect is small relative to the depletion effect.

At potentials below and above those sufficient for reduction, a current flows which is almost entirely due to capacity effects at the electrode surface (118). When the concentration of the depolariser is smaller than 10^{-5} M, the magnitude of the capacity current becomes comparable to that of the peak current and must be taken into account during measurement of the latter (118).

The theory of current-voltage curves for single sweep

oscillographic techniques has been worked out by Randles (119) and by Sevcik (120) for reversible electrode reactions. The expression derived by these authors for the peak current, ip, may be written as

$$i_p = Kn^{2/3} m^{2/3} t_p^{2/3} \alpha^{1/2} D^{1/2} C.$$

Where K is a numerical constant, n is the number of electrons involved in the reaction, m is the weight of mercury falling from the electrode per second, t is the time of drop life at which the sweep occurs, α is the rate of change of potential and c is the concentration of depolariser. Because of mathematical difficulties, the value of K could not be evaluated precisely, making a complete experimental check of this equation difficult. However, it has been shown experimentally that there is a linear relationship between ip and C for both inorganic and organic depolarisers (112, 119, 122).

The half-wave potential (Fig. 4) is characteristic of the material being reduced when polarograms are obtained by conventional methods. The potential at which the peak current occurs in oscillopolarograms is related to the half-wave potential by the equation (121)

$$e_p = E_{1/2} + \frac{29}{n}$$

where n is the number of electrons involved in the reduction of the depolariser.

Polarographic Behaviour of Azo Compounds

Aromatic azo compounds yield a single well defined re-

duction wave when polarograms are obtained using conventional techniques (110). This wave is attributed to the reduction of the azo group to the corresponding hydrazo derivative,

$$R\text{-}N = N\text{-}R + 4H + 2e \rightleftharpoons R\text{-}NH_2 - NH_2\text{-}R.$$

At a pH close to 8, the half-wave potential is approximately -0.5 volts with respect to the saturated calomel electrode (S.C.E.) (110).

The only systematic study of the oscillopolorograms given by azo compounds was made by Streuli and Cooke (122) using azobenzene. These workers found that the peak currents were considerably higher than those given by the same concentration of other depolarisers. This phenomenon was attributed to the adsorption of azobenzene on the mercury surface prior to the application of the sweep voltage, which was considered to lead to a higher concentration of the reducible material in this region than would normally be present.

The Effects of Adsorption

The adsorption of organic compounds on a mercury surface is a common phenomenon which has been studied extensively (123-126). In general, the surface active agent need not be capable of being reduced or oxidized at the potentials at which adsorption occurs.

When a non-reducible surface active compound is added to a solution of a depolariser, the peak (127) or diffusion current (128-129, 135) due to its reduction may decrease. Thus, caution must be exercised in using the peak height.

obtained in the presence of surface active agents to determine the concentration of depolariser. Since globular proteins and polypeptides are adsorbed on a mercury electrode (136-139), it may be necessary to introduce corrections to account for the decrease in current resulting from adsorption when polarograms are obtained in the presence of these materials.

The mechanism by which surface active agents decrease the magnitude of faradaic currents is not completely understood. However, it is usually considered that the decrease is not due to a diminution in the concentration of depolariser resulting from the formation of non-reducible complexes with the surface active material (123-126).

In addition to causing the effect on the faradaic current described above, the adsorption of surface active materials may result in the half-wave or peak potential being shifted into more negative values (138), or in alterations of the electrical capacity of the dropping mercury electrode, and, thus, of the capacity current (140). The changes in capacity current are easily measured and may be used to detect the adsorption of surfactants (140).

The capacity current at a dropping mercury electrode is given by the relation

$$i_c = C_d \frac{dE}{dT}$$

where i_c is the capacity current, C_d is the differential capacity per unit area and dE/dt is the rate of change of potential (140). The differential capacity, C_d is defined as dQ/dE, where Q is the charge per unit area and E, the potential. The capacity of the DME is defined as a differential since the integral capacity, Q/E, is a function of the potential. Since the rate of change of potential employed with a single sweep cathode ray polarograph is constant, according to the above equation, the current voltage curve obtained in the absence of depolariser is essentially a plot of the differential capacity as a function of potential. Loveland and Elving (141) have used this fact to study adsorption phenomena at the mercury surface with such instruments.

Polarographic Studies of the Binding of Small Molecules to Proteins

The application of polarography for measuring the binding of low molecular weight depolarisers with proteins is based upon the fact that the current due to the reduction of the depolariser decreases when it combines with protein (138). This decrease occurs either because the complex is not reducible, or because the complex yields a current with a half-wave potential indistinguishable from that of the free depolariser, but with a lower current per unit concentration (142). The diffusion current in such systems, i_d,

may be expressed as (142)

$$i_d = Mc + Nb \qquad (1)$$

where M and N is the current yielded per unit concentration of the free and bound depolariser, c and b, respectively. The values of c and b in these systems may be determined from diffusion current data by solving the two simultaneous equations represented by equation (1), and the relation

$$c_t = c + b \qquad (2)$$

In this latter relation c_t is the total concentration of depolariser.

In order to explain the current yielded by the protein-depolariser complex, it was suggested that (i) it is either reducible, or (ii) not-reducible, but that it may dissociate at the electrode surface (142). Two types of dissociable complexes were considered: one which dissociates at a rate yielding depolariser to the electrode at a rate comparable to that of diffusion, and one which dissociates much more rapidly. Complexes of the former type yield so-called kinetic currents which can easily be detected by their characteristic variation with the height of the mercury reservoir when polarograms are obtained by conventional methods (108). In oscillopolarograms, kinetic currents can be detected by the fact that they yield a current-voltage curve similar to that obtained by conventional techniques (121). In one study (142), two protein-depolariser complexes (bovine serum

albumin with Zn and Hg) were found not to yield kinetic currents, and it was concluded that these complexes were either reducible, or not reducible but capable of dissociating at the electrode surface at a very rapid rate.

In several systems, difficulties were encountered in using polarography to determine the amount of bound depolarizer because of complications arising from the adsorption of protein on the DME (138). The extent to which these complications occured seemed to depend upon the particular depolariser and protein under investigation as well as upon the pH of the solution (138). In systems where these complications did not occur, or where efforts were made to compensate for them, binding data could be obtained agreeing favorably with that obtained by the method of equilibrium dialysis (142, 143).

Polarographic Studies of Antibody Reactions

Breyer and Radcliffe (144, 145) used the polarographic method to study the combination of antibodies with their corresponding azo antigens, prepared by coupling diazotized p-aminobenzoic acid to egg albumin. The azo antigens were found to be reducible at a mercury electrode, and upon combination with their homologous rabbit anti-hapten antibodies, the diffusion current decreased. By studying the variation in current as a function of the relative concentrations of antibody and reducible antigen, it was shown,

in agreement with the results of the other workers (31-35) that rabbit antibody molecules had two combining sites.

An attempt to use polarography to measure the extent of reaction of the reducible hapten p-(2,4-dihydroxyphenylazo)-benzoic acid with its homologous rabbit antibodies was made by Saha and Chaudhuri (146). Unfortunately, it is likely that the results obtained by these authors represent artefacts. One reason for this is that many of the measurements were made with the whole antiserum instead of with the gamma-globulin fraction. Thus, it is probable that these measurements reflect primarily the interaction of the hapten with serum components other than antibodies, especially with serum albumin which is present in much higher concentrations than antibodies and which is known to react with azo compounds (147). Another reason is that in those instances where the gamma-globulin fraction of antisera was used, no attempt was made to distinguish between the effects on the faradaic current caused by the adsorption of these proteins on the DME and by the effects due to the reaction of hapten with antibodies. As indicated by the results of the present investigation (page 90) it is of critical importance to be able to make this differentiation in order to obtain meaningful results.

SCOPE OF THE PRESENT STUDY

The primary objective of the present study was to measure the rate of antibody-hapten interactions. At the time when it was initiated, and during its course, there were no kinetic data available for these reactions. However, from the existing kinetic studies of antibody-antigen reactions referred to in Chapter 1, it was suspected that they would be fast. Therefore, it was decided to resort to a sensitive physical method capable of following changes in free hapten concentration in the micromolar range over short intervals of time. In order to avoid complications resulting from the formation of large aggregates the reaction chosen for this study was that between univalent haptens and their homologous antibodies. In such systems only the bimolecular and termolecular complexes AbH and AbH_2 can be formed.

Oscillographic polarography, in conjunction with haptens containing reducible azo groups, was chosen to follow the reaction since this technique seemed to have the characteristics desired. In the present study, a method employing oscillographic polarography was developed to measure the binding of antibodies and their haptens in which only a small amount of antibody solution was required. The applicability of this method for the measurement of the extent of these reactions was evaluated by comparing the results given by the polarographic method with those given by equilibrium

dialysis. The speed of combination of antibodies with haptens as well as the rate of dissociation of the complexes was also investigated polarographically, the former by measuring the decrease in current caused by the formation of complexes, and the latter by following the increase in current resulting from the release of reducible hapten from the complexes when a non-reducible, inhibiting hapten was added to an equilibrated solution of reducible hapten and antibody.

CHAPTER 4

EVALUATION OF OSCILLOGRAPHIC POLAROGRAPHY FOR THE MEASUREMENT OF THE BINDING OF HAPTENS TO ANTIBODIES

Introduction

The rationale for using polarography for measuring the combination of a reducible hapten with its homologous antibodies was based on the assumption that the current due to reduction of hapten would decrease upon its combination with antibody. On this basis, the decrease in current would be expected to be proportional to the amount of hapten bound. However, proteins are surface active agents, and as such, may be adsorbed on the surface of the dropping mercury electrode, and thus, may decrease the faradaic current given by a depolariser (127-129, 135). Consequently, there was the possibility that on adding antibody to a solution of hapten, the decrease in current would be due to both of these phenomena instead of measuring only the extent of combination of the hapten with the antibody. Preliminary experiments indicated that this indeed was the case and, therefore, for the successful use of the polarographic technique in this study, it was essential to be able to evaluate the effect caused by the adsorption of proteins. Only then could one hope to measure quantitatively the portion of the decrease in current due to the combination of the reducible hapten with the antibody. In consequence, an appropriate

experimental method, to be described in detail, was developed for this purpose. The validity of the experimental procedures and of the method of treating the data was established by comparing the values of the intrinsic equilibrium constant and of the index of heterogeneity calculated on the basis of the polarographic experiments with those obtained by the method of equilibrium dialysis.

The antibody-hapten reactions studied in the present investigation consisted of the combination of the reducible haptens, p-(p-aminophenylazo)phenylarsonic acid and p-(2-4,dihydroxyphenylazo)phenylarsonic acid, with the antibodies specific to the p-(p-azophenylazo)phenylarsonate ion. These haptens were chosen since the polarographic behaviour of the reducible azo group is well known and is relatively uncomplicated, and also, since the reactions of the phenyl arsonate group with homologous antibodies have been exhaustively studied by many other workers (22, 27, 39, 72).

EXPERIMENTAL

MATERIALS

p-(p-aminophenylazo)phenylarsonic acid, (referred to hereafter as R^o)

The compound R^o was purchased from Aldrich Chemical Co., Milwaukee, Wisc. This product was found to be contaminated with some material which did not dissolve in 1 N NaOH and was purified by the following procedure during which it was converted to the hydrochloride salt. Three grams of the crude material were sus-

pended in 400 ml of water, the pH brought to 11 by the addition of NaOH and the insoluble fraction removed by centrifugation. The pH of the supernatant was then lowered to 4.5 in order to precipitate the acid form of R^{o}. The precipitate was recovered, redissolved at pH 11.5, and then reprecipitated at pH 4.5. This precipitate was dissolved in 3 liters of 60% ethanol (V/V), to which 100 ml. of concentrated hydrochloric acid was added: the ethanol was then removed by evaporation at room temperature by passing a current of air produced by a fan over the surface of the solution. The product which crystallized out was washed with ethanol and dried in vacuo. The hydrochloride derivative was found to be at least 98% pure by titration with NaOH.

p-(2,4-dihydroxyphenylazo)phenylarsonic acid (referred to hereafter as Res-R)

The compound Res-R was prepared by the addition of diazotized arsanilic acid (149) to a 100 fold molar excess of resorcinol in one molar Na_2CO_3. Purification was accomplished by repeated precipitation from alkaline solutions by the addition of acid, followed by recrystallization from ethanol. Titration of the azo group with titanous chloride (148) indicated that the product was at least 97% pure.

Preparation of Soluble Hapten Protein Conjugates

The diazonium salt of R^{o} was prepared by the addition of an alkaline solution containing the amine and sodium nitrite to a solution of 1 M HCl. This method was found to be more

convenient than the procedure employed by Pauling et al. (149) for the diazotization of R^o because of the low solubility of this compound in acid solutions. For the diazotization, 0.89g of the hydrochloride of R^o and 0.176g of sodium nitrite were dissolved together in 80 ml. of 0.2 M NaOH, and slowly added to 30 ml of HCl at 5-10°C.

Conjugates of R^o with bovine gamma globulin (BGG) and rabbit serum albumin (RSA) were prepared by the addition of diazotized R^o to a solution of the protein at pH 10. One hundred moles of the diazonium salt of R^o were added for each mole of protein in solution. After allowing the reaction mixture to stand for 24 hours at 4°C., low molecular weight by-products were removed by exhaustive dialysis against large volumes of borate-NaCl buffer at pH 8.0 and ionic strength 0.15. Complete removal of colored by-products required at least 3 to 4 weeks of continuous dialysis of 4°C. When colored material stopped diffusing out of the dialysis bags, the solution of BGG-R^o conjugates was dialysed against 0.15 NaCl and then Seitz-filtered into sterile vials. The corresponding RSA-R^o conjugates were dialysed against water and then lyophilized, since storage of this test antigen in the dark, under sterile conditions at 4°C, resulted in the formation of dialysable, colored products, which might have inhibited the antibody-hapten reaction.

Immunization of rabbits

Antibodies specific to the p-(p-azophenylazo)phenylarsonate ion were prepared by the immunization of rabbits with conjugates of R^{o} and bovine gamma globulin. The presence of antibodies to the hapten in the immune sera was detected using conjugates of R^{o} and rabbit albumin. The rabbits were immunized by intravenous injection into the external marginal ear vein 3 times a week for a period of 4-6 weeks with 1 ml. portions of a 2% solution of the antigen until sufficiently high titer antisera were produced. They were then 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°C.

Fractionation of Antisera

All experiments were carried out with gamma-globulin fraction of pooled antisera, isolated by salt-precipitation according to the method of Marrack et al. (150). The original procedure was slightly modified, inasmuch as the precipitated protein was dissolved in borate-NaCl buffer (pH 8.0, u = 0.15) and not in phosphate buffer. After completion of the fractionation procedure, the globulin solutions were dialysed extensively at 4°C against the borate-NaCl buffer and then Seitz-filtered into sterile vials.

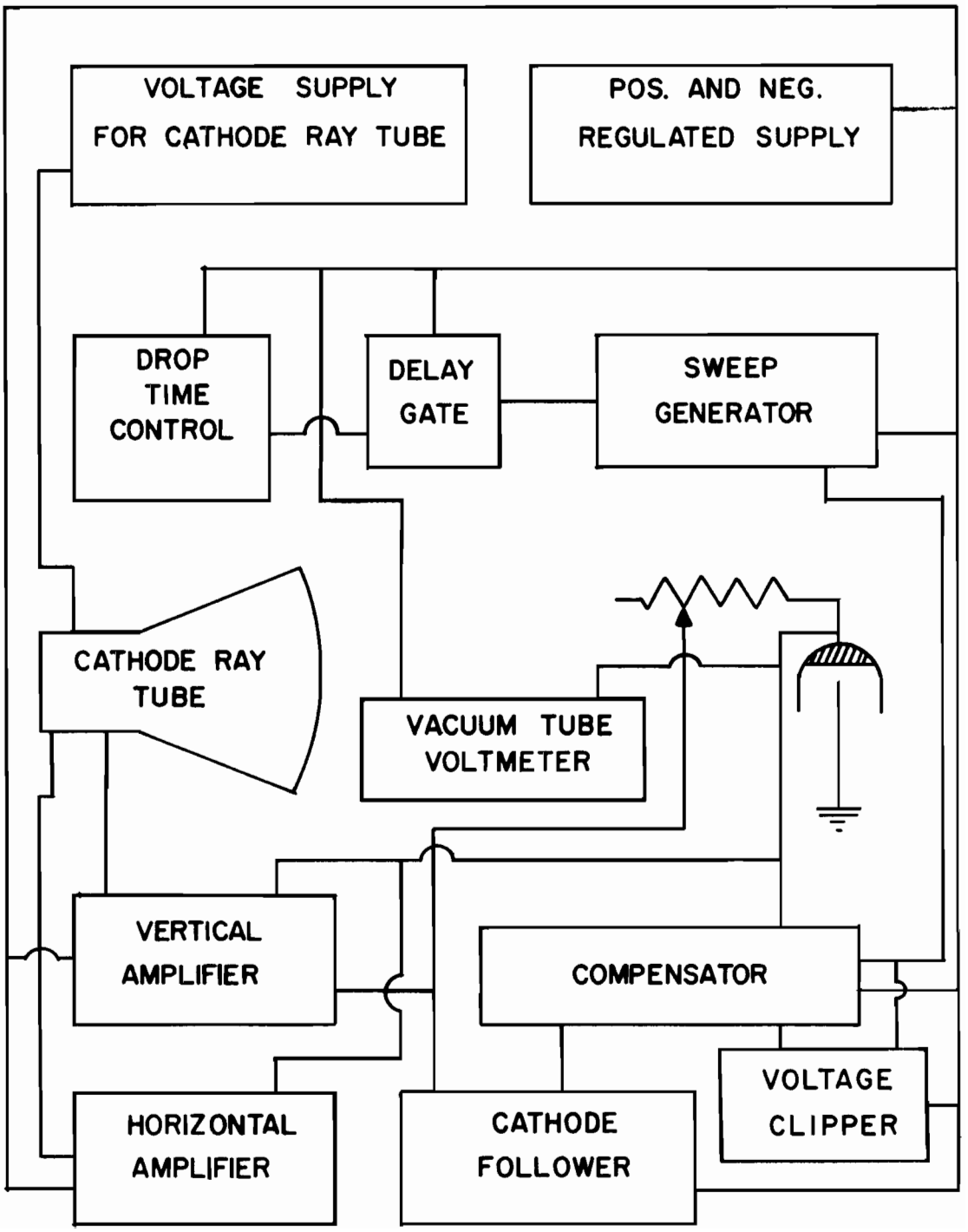
Two pools of antisera were used in the experiments with R^{o} and Res-R, and these are referred to as B-50(5) and B-50(6),

FIGURE 5

(opposite side)

FIGURE 5

BLOCK DIAGRAM OF THE OSCILLOGRAPHIC POLAROGRAPH



respectively. It was necessary to use pools of antisera because the amount of antibody required was much larger than that which could be obtained from one, or from several bleedings from a single rabbit.

Normal gamma globulins were isolated from pooled normal rabbit serum using the same procedure employed for immune globulins.

Borate Buffer

The borate-NaCl buffer, pH 8.0, μ = 0.15, in which all experiments were carried out, was prepared by adding 0.15 M NaOH to a solution containing 0.2 M H_3BO_3 and 0.15 NaCl until the desired pH was obtained.

THE CATHODE RAY POLAROGRAPH

The cathode ray polarograph (C.R.P.) employed was a modified version of that designed by Snowden and Page (112). A block diagram of this modified instrument is shown in Fig. 5. The sequence of events which occured during its operation may be summarised as follows:

(1) The drop-time control knocked the mercury drop off the electrode and at the same time caused the delay gate (i) to switch off the voltage sweep generator, and (ii) to start a timing circuit which determined when a new sweep was to take place (delay time).

(2) At the end of the delay time, the delay gate switched on the sweep generator.

(3) The sweep generator produced a linear voltage sweep.

(4) This voltage sweep was acted upon by the voltage clipper and the compensator, and then applied to the cell.

(5) The voltage sweep was amplified and applied to the plates of the CRT while, simultaneously, the voltage drop across the resistor in series with the polarographic cell was amplified and applied to the vertical plates.

(6) The drop-time control knocked the mercury drop off the electrode and at the same time activated the delay gate, thus causing step 1 to be repeated.

The factors which could be varied in this sequence were:

(a) the delay time (1-7 secnnds)
(b) the drop time (1-7 seconds)
(c) The magnitude of the voltage sweep (0.5 to - 2.5 volts)
(d) the rate of change of sweep voltage (1-100 volts/sec.)
(e) size of series resistor (0 to 100,000 ohms)
(f) gain of horizontal and vertical amplifiers.

The modifications to the design of the Snowden and Page C.R.P. were introduced for the purpose of the present investigation and consisted of minor changes in circuit elements such as tubes, resistors, and condensers, and of the addition of the drop-time control to synchronise the start of the voltage sweep with the mercury drop. The reasons for making them are given below. None of these modifications altered substantially the mode of operation of the circuits employed by Snowden and Page (112). The complete circuit diagrams are shown in Figs. 6 to 10.

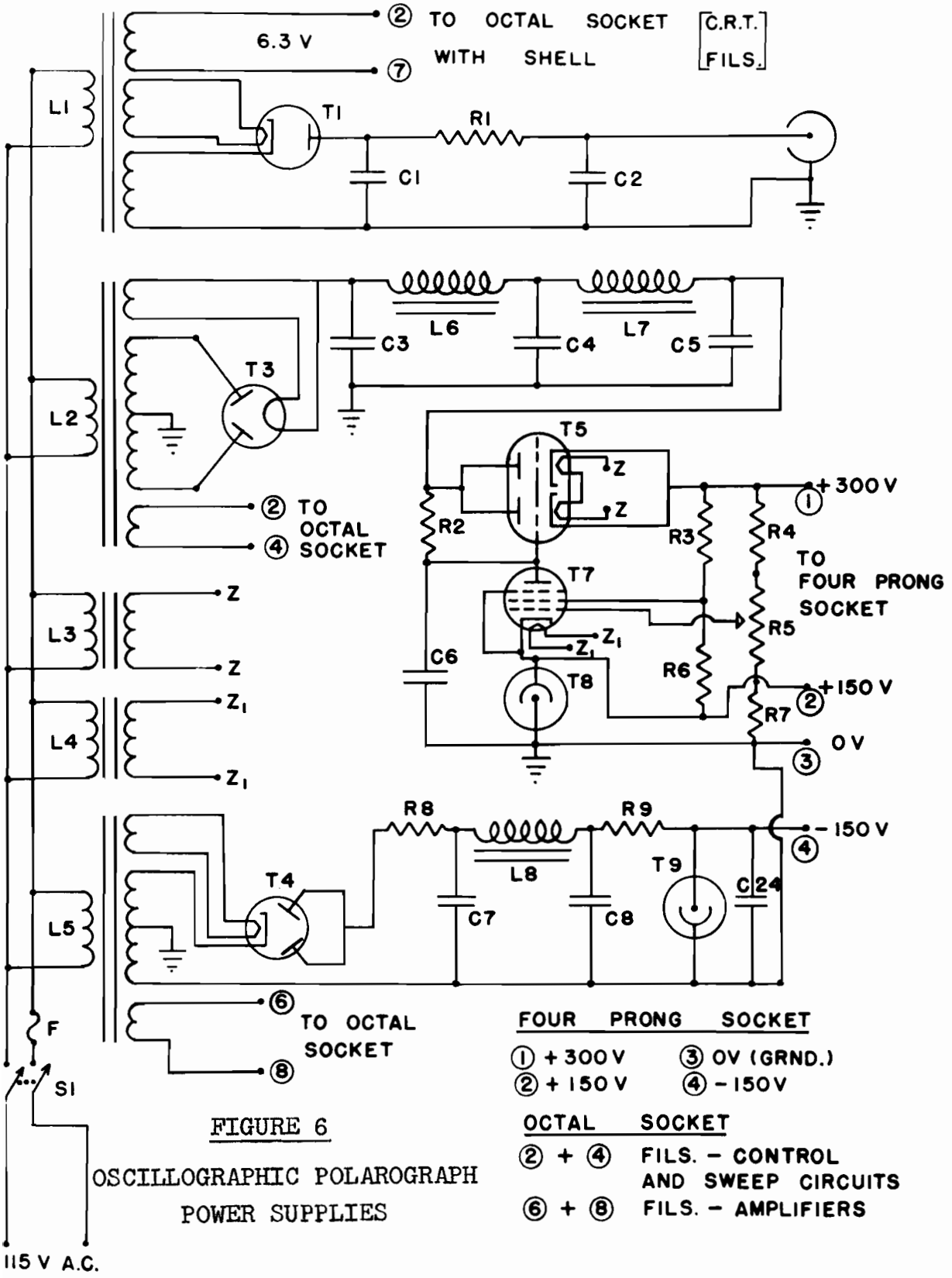


FIGURE 6

OSCILLOGRAPHIC POLAROGRAPH POWER SUPPLIES

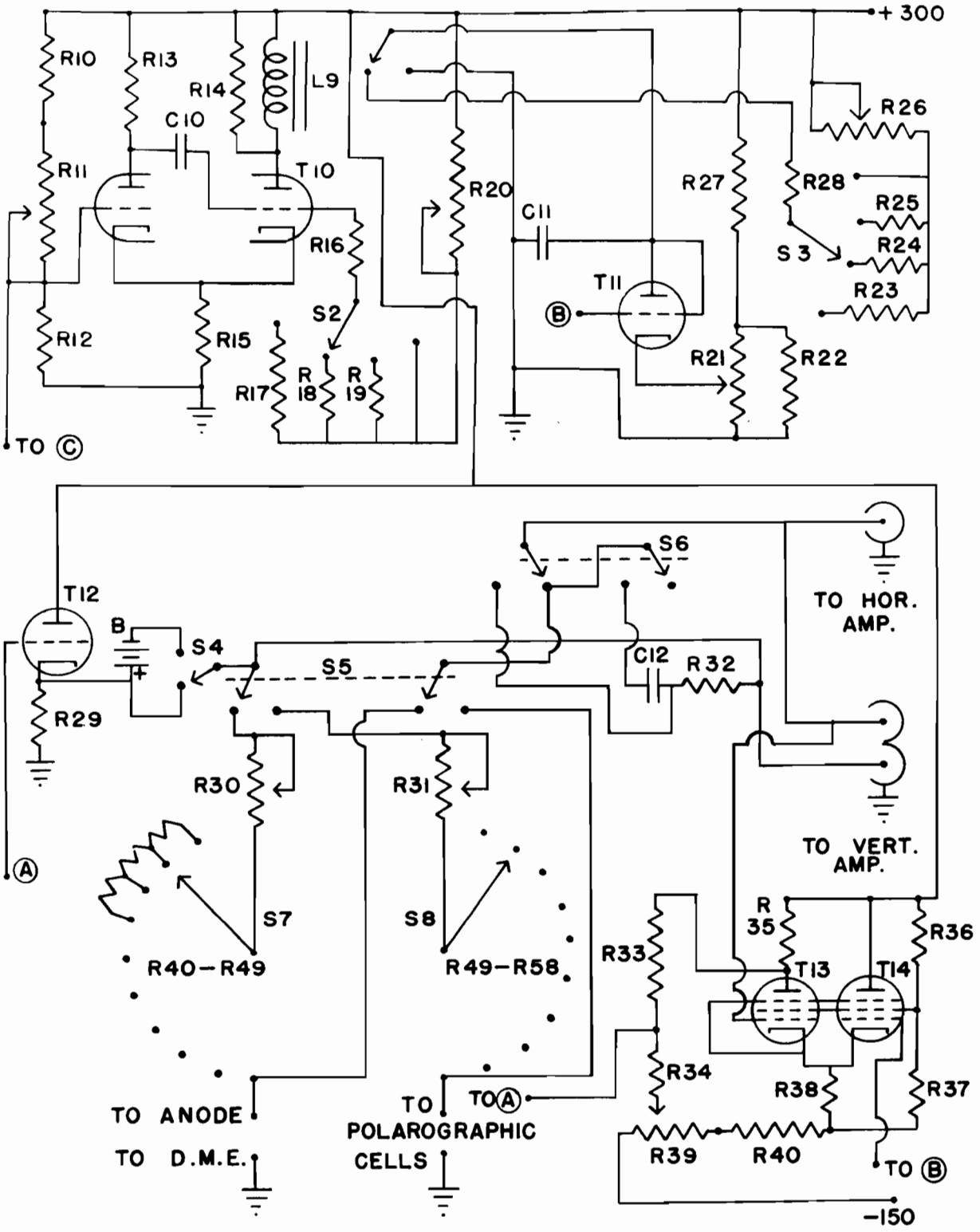


FIGURE 7

OSCILLOGRAPHIC POLAROGRAPH

CONTROL CIRCUITS

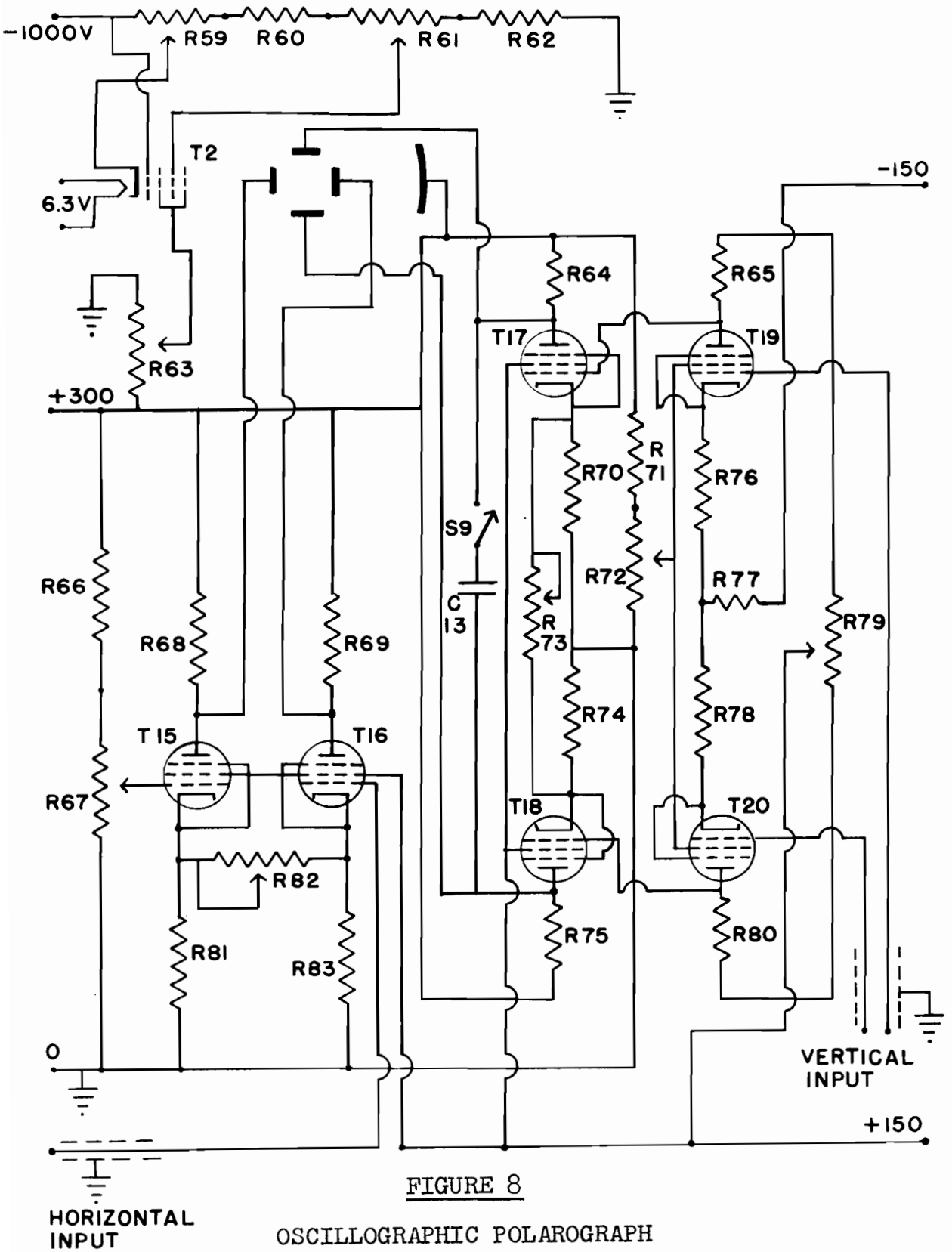


FIGURE 8

OSCILLOGRAPHIC POLAROGRAPH AMPLIFIERS

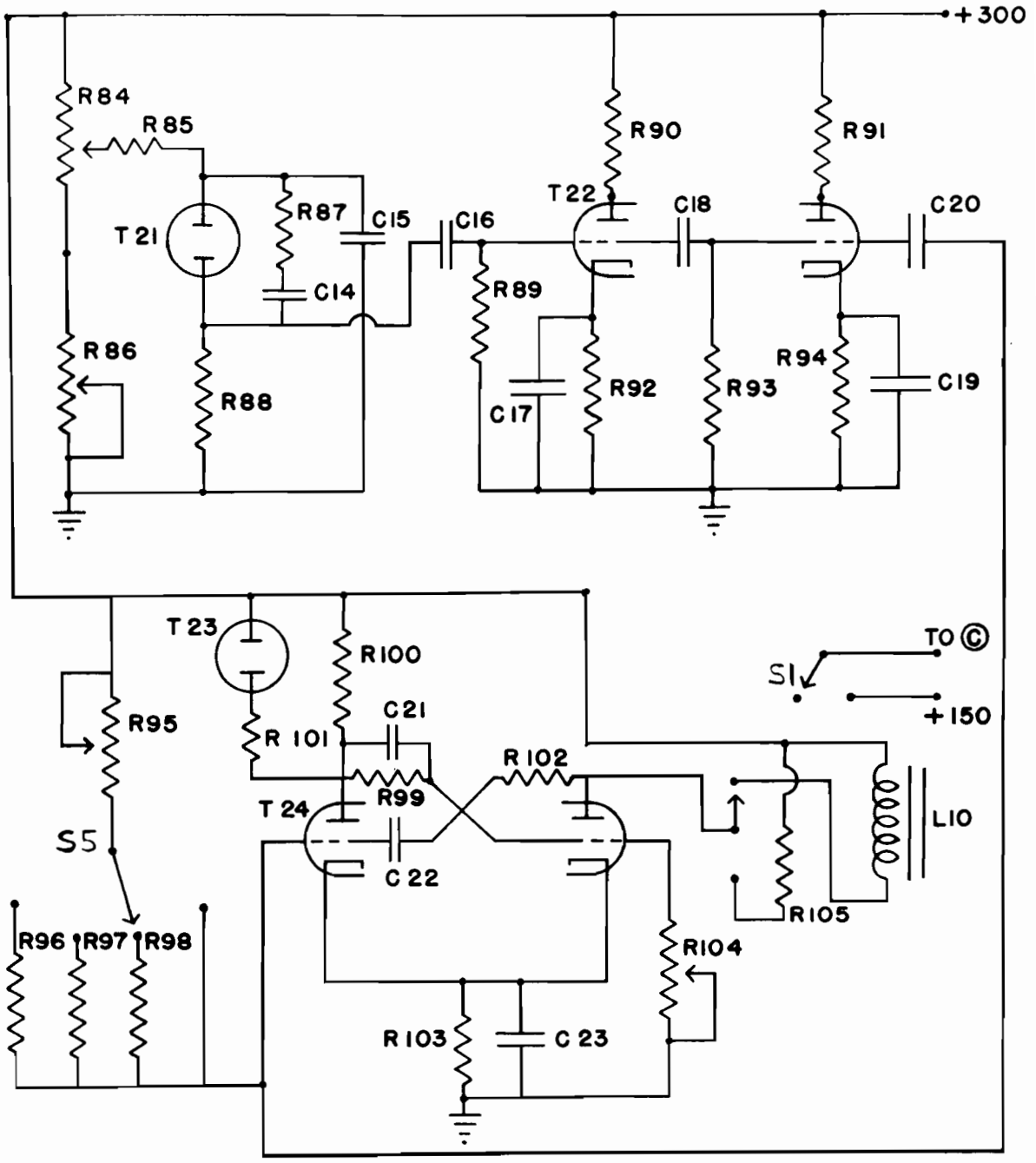


FIGURE 9

OSCILLOGRAPHIC POLAROGRAPH
DROP TIME CONTROL

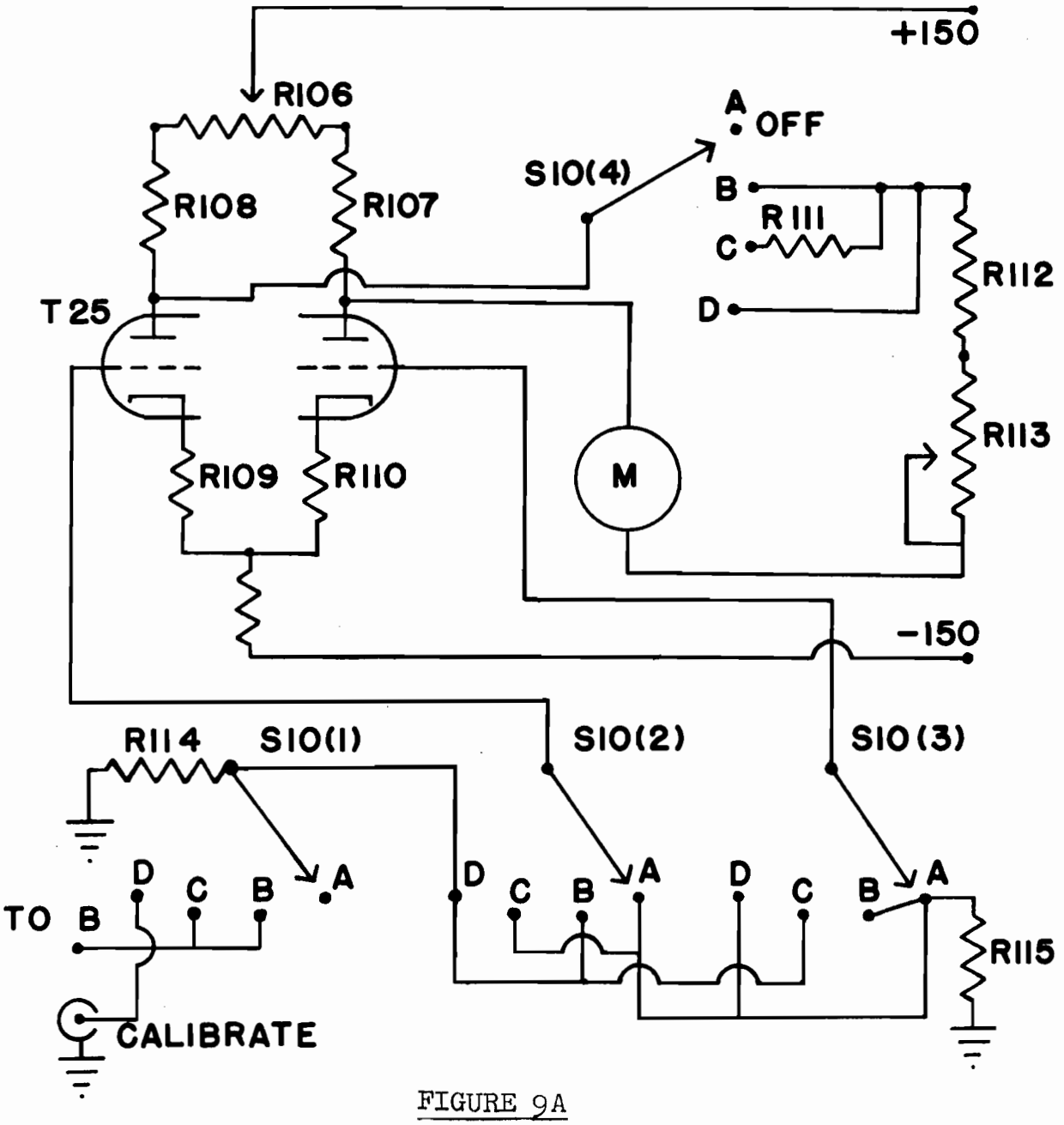


FIGURE 9A

OSCILLOGRAPHIC POLAROGRAPH
VACUUM TUBE VOLTMETER

FIGURE 10

LIST OF COMPONENTS

B	9-volt C battery
C1,C2	0.1 μfd; 4KV
C3	8 μfd; 600V
C4	16 μfd; 800V
C5	0.5 μfd; 600V
C7,C8	8 μfd; 600V
C10	1 μfd; 600V
C11	0.47 μfd; 600V
C13	0.05 μfd; 600V
C14	0.01 μfd; 600V
C15	1 μfd; 600V
C16	0.04 μfd; 600V
C17,C19	1.0 μfd; 600V
C18	0.04 μfd; 600V
C20	0.008 μfd; 600V
C21	25 μμfd; 600V
C22	0.47 μfd; 600V
C23	0.01 μfd; 600V
L1	Transformer, 1600V secondary (Triad, R-43C)
L3	Transformer, 375-0-375V secondary
L3,L4	Transformer, 6.3V secondary
L5	Transformer, 375-0-375V secondary
L6,L7	10 henries, 150 ma.
L8	25 henries, 40 ma.
L9,L10	Relay, 10,000 ohm coil (Potter and Bremfield, type LM11)

List of Components
Figure 10 (con't)

M	Microammeter, 100 μamps. sull scale.
R1	100K, 5 watts
R2	600K
R3	5K, 10 watts
R4	100K
R5	50K, 4 watts
R6	1500K, 4 watts
R7	100K
R8	2.5K
R9	2.8K
R10	1.2M
R11, R12	1M
R13	20K
R14, R15	10k
R16	2.2M
R17,R23	30M, precision, 1%, 1W.
R18, R24	20M, precision, 1%, 1W.
R19, R25	10M, precision, 1%, 1W.
R20, R26	10M
R21	50K
R22	20K
R27	270K
R28, R29	1K
R30	10K Helipot, 0.05% linear
R31	10K Helipot, 0.01% linear
R32	3.9M
R33	5M
R34	2.2M
R35	10K
R36	60K
R37	47K

List of Components
Figure 10 (con't)

R38	470
R39	50K, 4 watts
R40-R58	10K, precision resistors 1% tolerance, 1 watt
R59	50K, 4 watt
R60	150K
R61	250K, 4 watt
R62	600K
R64	100K
R65	330K
R66	1.5M
R67	100K, 4 watt
R68, R69	220K
R70	10K
R71	68K
R72	40K, 4 watt
R73	1K, 4 watt
R74	10K
R75	100K
R76, R78	10K
R79	15K, 4 watt
R80	330K
R81	10K
R82	1K, 4 watt
R83	10K
R84	1M
R85	24M, 1/2 watt
R86	200K
R87, R88	1K
R89	4.7M
R90, R91	0.47M
R92, R94	6.8K

List of Components
Figure 10 (con't)

R93	2.2M
R95	10M
R96	30M
R97	20M
R98, R100	10K
R99	220K
R101	560K
R102	0.22M
R103	10K
R104, R105	1M
R106	2K
R107, R108	10K
R109, R110	4.7K
R111	2K
R112	12K
R113	20K, 4 watt
R114, R115	5M
S_1	Relay contacts on L10
S_2,S_3,S_5	Rotary switch, 1 pole, 5 position
S_4	S.P.S.T. toggle switch
S_5	Rotary switch, 1 pole, 5 position
S_6	DPOT toggle switch
S_7,S_8	Rotary switch, 1 pole, 11 position
S_9	S.P.S.T. toggle switch
S_{10}	Rotary switch, 4 gang, 4 pole, 5 position.
T1	2X2A
T2	5ADP7

List of Components
Figure 10 (con't)

T3	5Y3
T4	5Z4
T5	6080
T6	
T7	65S7
T8, T9	OD3
T10	6SN7
T11, T12	6J5
T13, T20	6SJ7
T21, T23	NE2
T22	6SL7
T24, T25	6SN7

Modifications

(i) Drop-time Control

In the original design of Snowden and Page (112), the delay gate was triggered by the fall of the mercury drop. When the drop fell off the electrode, it caused a large change in the current flowing through the cell and series resistor which gave rise to a pulse in the output of the vertical amplifier. This pulse was used to trigger the delay gate. In the early stages of this study the use of this method was found to be very tedious since the setting of a potentiometer (multivibrator sensitivity, R 11) controlling the delay gate had to be altered whenever the concentration of depolariser was increased. In addition, proper synchronisation could not be achieved with drop times smaller than two seconds. The drop-time control was installed to avoid these difficulties.

The fundamental part of the drop-time control circuit (Fig. 9) was a neon bulb oscillator operating at a frequency (0.1 to 1 cycles per second) so that the condensor, C 14, was discharged at the time it was desired to dislodge a drop from the mercury electrode. The circuit was made to operate in the following way. Upon discharge of the condensor, the pulse produced was amplified and then used to trigger a one-shot multivibrator (T 22). When the multivibrator was triggered the relay in one of its plate circuits closed. The armature of the relay was

connected to the dropping mercury electrode by means of a stiff copper wire so that whenever the relay was made to operate, a drop was knocked off the electrode. Simultaneous triggering of the delay gate and dislodgement of a drop from the DME was accomplished by using the contacts of the relay to apply -150 volts to the control grid of the multivibrator comprising the delay gate (labelled T 10 in Fig. 8).

(ii) Minor Circuit Changes

Cathode Ray Tube Circuits

(a) A 5ADP7 cathode ray tube was substituted for the 5LP7 tube used by Snowden and Page. The former had a flat face so that parallax errors in measuring the peak height were minimized.

(b) An astigmatism control (152) was added to the CRT circuit since otherwise the spot could not be focused properly.

(c) A 1600 volt transformer was used to supply the accelerating voltage since the type used by Snowden and Page (1800v) was no longer available commercially.

(d) The blanking relay used by Snowden and Page (112), which prevents the trace from appearing on the face of the CRT until the sweep voltage is applied to the cell, was eliminated from the circuit since it was found to be unnecessary.

Power Supplies

(a) The two 2A3 tubes were replaced with a single 6080 tube. This latter tube fulfills the same function as the

former two, but is more reliable.

Control Unit and Sweep

(a) The magnitude of the resistors R20 - R22, R28 and of the condensor C11 used in the modified instrument were different from those used by Snowden and Page. These changes were necessary since the delay time and voltage sweep of interest could not easily be obtained unless these substitutions were made, presumable due to slight differences in the characteristics of the tubes used in the two instruments.

(b) A switch was installed which permitted the removal of the battery B from the circuit and its replacement by a direct connection. One function of this battery was to permit the voltage sweep to start from positive values. However, to avoid difficulties resulting from varying starting voltages caused by thermal or aging effects on the voltage of the battery, the switch was installed. The starting potential was zero volts when the battery was removed from the circuit.

(c) The magnitude of the resistors in series with the polarographic cell was increased from 10,000 to 100,000 ohms since it was anticipated that lower currents would be measured in this study than is ordinarily done.

Cathode Ray Tube Amplifiers

(a) A 500 ohm potentiometer was placed across the 6.3 V line supplying the filament voltage to tubes of the C.R.T. amplifiers and its center tap connected to ground in order to minimize hum caused by the filament circuit.

PROCEDURE FOR EQUILIBRIUM POLAROGRAPHIC EXPERIMENTS

The sample of the protein solution was placed in the polarographic cell and deoxygenated as described below. Microliter increments of 1.04×10^{-3} molar solutions of the reducible hapten, previously freed of oxygen, were then added at intervals of about two minutes. After each addition the solution was stirred with a magnetic stirrer for 30 to 60 seconds, and the polarographic tracing was recorded for the quiescent solution (which was within 30 to 60 seconds after the stirrer was stopped). The drop time used in these experiments was 5 to 7 seconds, the delay time 4.5 seconds, and the sweep rate 10 volts/second. All polarographic experiments were made at $25 \pm 0.1°C$.

Polarographic Cell Assembly

A diagram of the polarographic cell assembly, is shown in Fig. 11. The cell was a specimen vial (14 mm i.d. and 60 mm high) and was immersed to a depth of 4 cm in a thermostat during an experiment. One ml of antibody solution or normal gamma-globulin was used for each run in which the DME was immersed to a depth of 3-4 mm. The natural drop time of the DME in the buffer or in the protein solutions was 5-7 seconds. The capillaries used for the DME were obtained from E. H. Sargent and Co., Chicago, Illinois. The non-polarizable anode was silver-silver chloride electrode consisting of a silver wire (1 mm dia.) encased in a

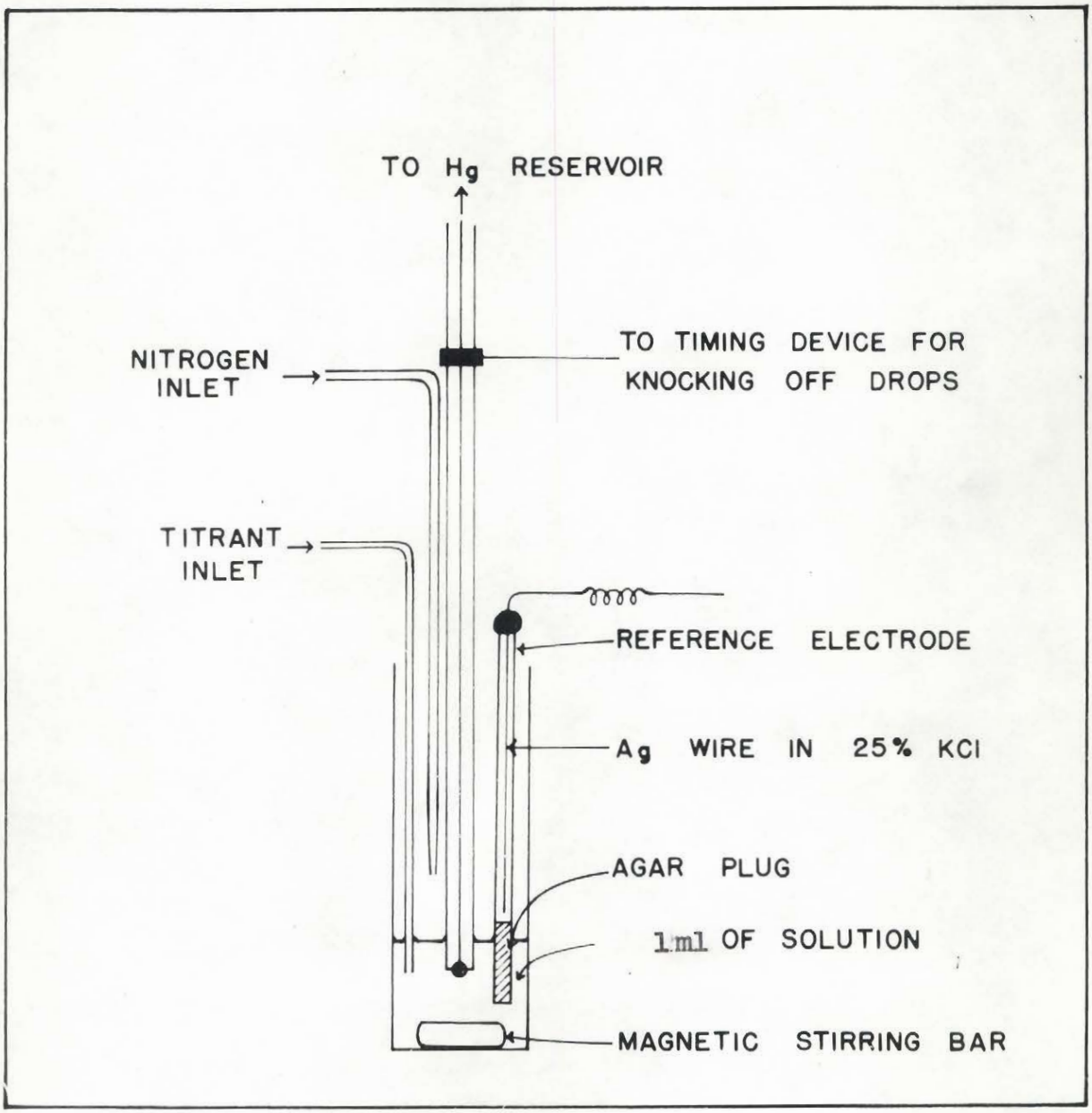


FIGURE 11

POLAROGRAPHIC CELL ASSEMBLY

polyethylene tube (1-5 mm i.d.) which was filled with a chloride solution and plugged at the bottom with agar gel. The chloride solution was either 25% KCl or 0.15 M KCl, and was also used for making the agar gel plug. The potential of the silver-silver chloride electrodes with respect to the saturated calomel electrode were -0.053 and -0.106 volts, respectively.

Hapten solutions were added to the cell using an "Agla" micrometer syringe (Burroughs Wellcome Co. Ltd., London, England) calibrated in units of 0.2 microliters, which could be read to 0.01 microliter. The hapten solution was delivered from the micrometer syringe into the solution in the polarographic cell through a thin (1 mm i.d.) polyethylene tube.

Deoxygenation of Solutions

Protein solutions were deoxygenated by passing a gentle stream of purified nitrogen over their surface for 10-15 minutes while agitating simultaneously with a magnetic stirrer. The nitrogen was led into the cell by means of two teflon tubes (1.5 mm i.d.), one of which was kept at a distance of 5 mm above the surface, and the other at a distance of 30 mm. The rate of flow of nitrogen was the same in both tubes and was such that the meniscus was only slightly deformed at the point where the gas from the lower tube impinged on the solution. Complete deoxygenation was almost invariably achieved with this procedure within ten

minutes, as indicated by the absence of peak currents due to the reduction of oxygen in oscillopolarograms obtained after this time.

During the deoxygenation procedure the non-polarizable electrode was kept in the protein solution. However, the DME was placed in the cell and immersed in the solution only after complete deoxygenation had been achieved in order to avoid contamination of the solution with mercuric ions as a result of the reaction of metallic mercury with chloride ions in the presence of oxygen (108).

The nitrogen employed was the ordinary commercial product and any traces of oxygen were removed by passing it through three bubblers filled with an acidic solution of chromous sulfate (108). This solution was prepared by the reduction of chromic sulfate with zinc amalgam in one molar sulfuric acid (108). This solution, when fresh, imparted a garlic-like odor to the nitrogen passing through it. The odorous gas, thought to be arsine resulting from the reduction of arsenic present as an impurity in the zinc (153), was removed by passing the gas through a bubbler filled with alkaline potassium permanganate.

To prevent either evaporation of the solution or condensation of water vapor from the nitrogen during deoxygenation, it was necessary for the nitrogen to contain an appropriate amount of water vapor. Experience revealed that volume changes could be kept to within 1% when the deoxygenated nitrogen was passed through a bubbler filled with a borate

NaCl buffer (prepared by dissolving 0.2 moles of boric acid, 0.03 moles of sodium hydroxide, and 1.15 moles of sodium chloride in 1 liter of water), and then through 1 mm capillary tubes leading to and from a 125 Erlenmeyer flask; the bubbler and the flask were immersed in a thermostat maintained at 25°C.

Hapten solutions were deoxygenated by passing nitrogen through them for 5 minutes. These solutions were then introduced into the micrometer syringe. During the passage of nitrogen through the hapten solution the syringe was alternately filled and emptied with the hapten solution in order to remove the air contained in it.

Photographic Recording of Oscillograms

Oscillographic polarograms were recorded with a 35 mm Minolta, single lens reflex camera. The shutter of the camera was opened 0.5 to 1 second prior to the beginning of the voltage sweep and closed immediately afterwards. Kodak Plus-X and Tri-X film was used, with f-stops varying from 2 to 5.6, depending upon density of the negatives desired.

Measurement of Peak Height

The peak height in each oscillogram was determined by superimposing the pattern obtained in the presence of hapten upon the pattern obtained in its absence (capacity curve), and then measuring the distance from the apex of the peak to the point on the capacity curve corresponding to the

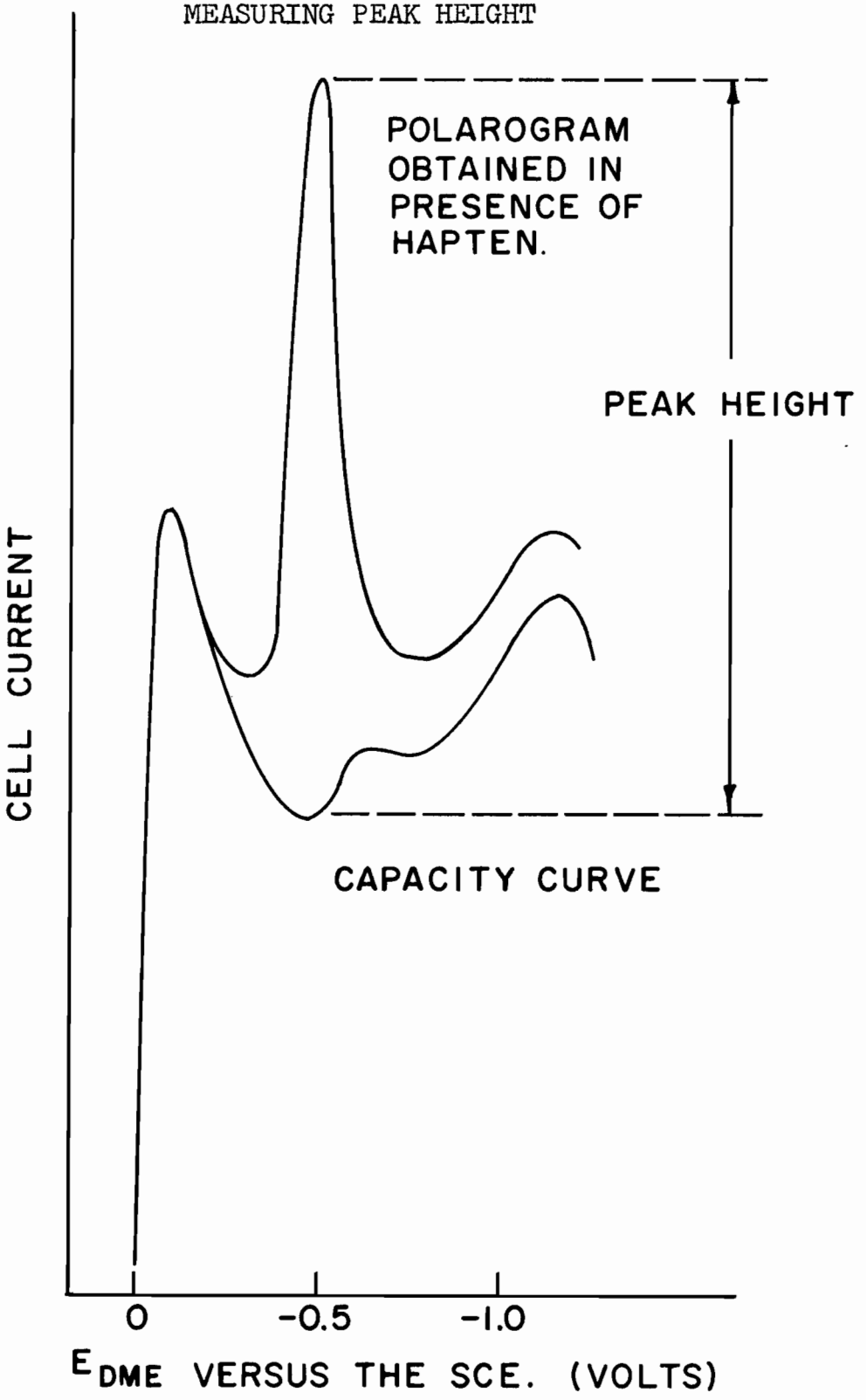
potential of the peak. This procedure is illustrated in Fig. 12 and actual photographs of oscillograms showing typical polarographic patterns are shown in Fig. 13. The height of the peak was measured in all cases in terms of the distance between the horizontal lines appearing on photographs of the CRT traces (Fig. 13). These lines were produced by an edge-lighted graticule kept in front of the cathode ray tube. The distance between the horizontal lines, which is referred to as a peak height unit, was about 0.2 in. and represented about 3.3 μamps under the conditions usually employed.

Procedure Used For Equilibrium Dialysis Experiments

Equilibrium dialysis experiments were carried out using a technique similar to that employed by Nisonoff et al. (84). One ml of the antibody solution was placed in a dialysis sac made from 1/4" i.d. Visking cellulose tubing (Visking Co., Chicago, Illinois) and then equilibrated against an equal volume of hapten solution contained in a four ml screw-cap vial, the cap of which was lined with parafilm. Equilibrium was achieved within 12 hours at 25°C when the vials were rotated at 2 to 3 R.P.M. All cellulose tubing used for dialysis membranes was treated according to the procedure described by Hughes and Klotz in order to remove soluble impurities (154).

After equilibrium had been achieved, the concentration of hapten in the solution which did not contain protein was

FIGURE 12
ILLUSTRATION OF PROCEDURE USED FOR
MEASURING PEAK HEIGHT



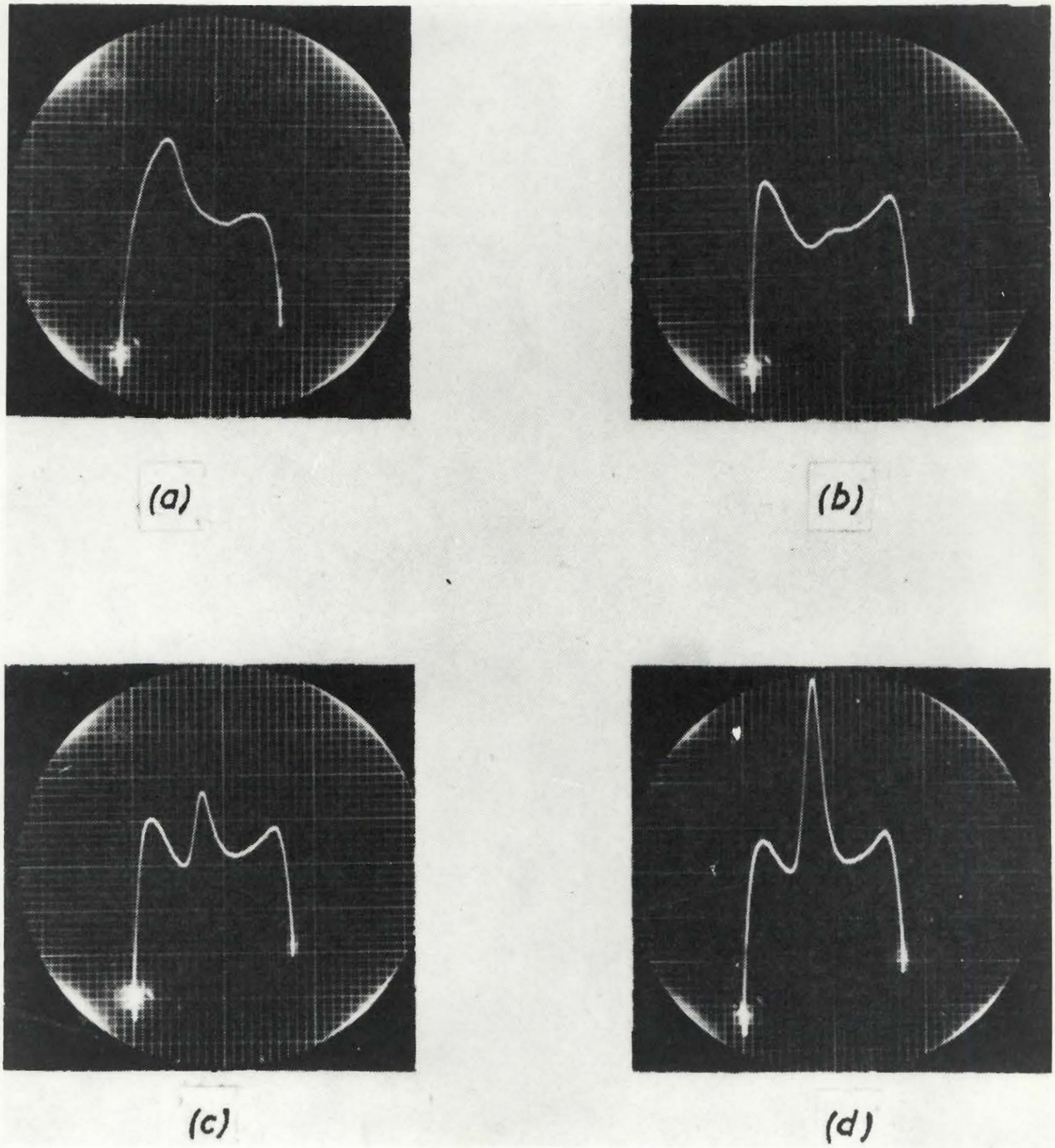


FIGURE 13

OSCILLOGRAPHIC POLAROGRAMS

Curves (a) and (b) represent oscillographic traces for the buffer and antibody solutions in the absence of hapten: curves (c) and (d) represent the traces obtained when increasing amounts of reducible hapten were added to the antibody solution.

determined spectrophotometrically at the wavelength of its maximum absorption in the near u.v., 387 mμ for R^{o} and 435 mμ for Res-R. This concentration corresponds to the concentration of free hapten in the protein compartment and is referred to as [c].

The number of hapten molecules bound by the antibodies in the protein compartment was computed with the aid of the relation

$$H_b = H_t - V_t [c] N - H_c \qquad (1)$$

where H_b = number of hapten molecules bound by the antibody
H_t = total number of hapten molecules in the system
[c] = the concentration of free hapten
V_t = total volume of solution in the cell
H_c = number of hapten molecules bound non-specifically
N = Avogadro's number

and then the concentration of the bound hapten, b, was determined using the relation

$$b = \frac{H_b}{N.V_p} \qquad (2)$$

where V_p is the volume of the protein compartment

The total volume of solution in the cell, V_t, was taken to be equal to the sum of the volumes of the protein and hapten solutions added plus the volume of liquid introduced into the system by the cellulose tubing, which had been soaked in the buffer solution prior to assembling the cell. The latter

volume amounted to 0.15 ml, as determined by weighing the membrane while wet, and then after drying at room temperature. The volume of the protein compartment, V_p, was calculated on the basis of the known volume of the protein solution used to fill the dialysis bag and the initial and final protein concentration, as determined by optical density measurements at 280 mμ*.

By using equation (1) to calculate the number of hapten molecules in the protein compartment bound by antibodies, cognizance was taken, through the presence of the term Hc, that some hapten may have been bound non-specifically by the membrane, by non-antibody protein ("normal gamma globulin") or by both. Preliminary experiments indicated the haptens were bound by both the membrane and normal gamma globulins. The binding of hapten by the membrane was indicated by the fact that the optical density of hapten solutions decreased when strips of Visking tubing were soaked in them for 12 hours. The binding to normal gamma-globulin was demonstrated by the fact that in equilibrium dialysis experiments with this material, the amount of hapten bound was significantly larger than that which could be attributed to binding by the membrane. The amount of hapten bound non-specifically at various concentrations of free hapten was

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* The final concentration of protein was found to be about 10% higher than the initial value, presumably owing to the fact that the bags were filled in such a way as to contain an air bubble at a pressure somewhat greater than that of the atmosphere. It was found necessary to include this air bubble since the bags could not otherwise be filled properly.

determined by equilibrium dialysis experiments in which normal gamma-globulin was substituted for the antibody solution with the aid of the relation

$$H_c = H_t - V_t [c] N \qquad (3)$$

The concentration of hapten bound only to normal gamma-globulin, Hn, was determined in equilibrium dialysis experiments from measurements of the difference in optical density between the two compartments. This determination depends on the fact that the optical density of the compartment containing only buffer, OD_1, may be written as

$$OD_1 = \epsilon_1 c \qquad (4)$$

where ϵ_1, is the extinction coefficient of the free hapten, while that of the compartment containing normal gamma-globulin is

$$OD_2 = \epsilon_1 c + \epsilon_2 H_n \qquad (5)$$

where ϵ_2 is the extinction coefficient of hapten bound to normal gamma-globulin. Accordingly,

$$OD_2 - OD_1 = \Delta OD \qquad (6)$$

$$= \epsilon_2 H_n$$

$$\text{and } H_n = \frac{\Delta OD}{2} \qquad (7)$$

Evaluation of Hn from equation (7) requires that ϵ_2 be known. The value for this extinction coefficient was taken to be equal to that of the free hapten, the justification being that the absorption spectrum of the haptens in solutions

of normal gamma-globulins containing up to two times the amount of protein used in the actual binding experiments (i.e., up to 18 mg/ml) had absorption specrra indistinguishable from those obtained in protein-free solutions.

Determination of Intrinsic Equilibrium Constant and Concentration of Antibody Sites

In systems where the antibody-hapten reaction appeared to be characterized by a single intrinsic equilibrium constant, the value of this quantity as well as the concentration of antibody sites, Ab, was determined graphically with the aid of the equation (84)

$$\frac{1}{b} = \frac{1}{c}\ \frac{1}{Ab\ K} + \frac{1}{Ab}$$

from the plot of the reciprocal of the concentration of bound hapten, 1/b, versus the reciprocal of the concentration of the free hapten, 1/c. For heterogeneous systems, the equation of Sips (84)

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

was employed to evaluate the average intrinsic equilibrium constant, K_o, the index of heterogeneity, a, as well as the concentration of antibody sites. This was done by plotting 1/b versus $\frac{1}{(c)^a}$ for the value of a which linearized the data, and then computing Ab and K_o from the slope and appropriate intercept.

RESULTS AND DISCUSSION

Part 1 - Equilibrium Dialysis - Characterization of the Binding of R^o and Res-R by the Homologous Antibodies

Binding curves obtained by the method of equilibrium dialysis for the reaction of R^o with the antibodies in B-50(5) and for Res-R with the antibodies in B-50(6) are shown in Figs. 14 and 15, respectively. The average intrinsic equilibrium constants and antibody concentrations computed from these curves as well as other related data are summarized in Table II. As mentioned previously, the reason for carrying out the equilibrium dialysis experiments was to be able to compare the results obtained by polarography with those given by a method known to yield accurate results.

TABLE II

Binding Data Obtained by Equilibrium Dialysis

System	Protein Conc. (mg/ml)	Antibody Conc. (M) x 10^{+5}	Intrinsic Eqlm. Const. (1/mole) x 10^{-5}	Index of Heterogeneity
R^o and B-50(5)	10.28	1.19	8.30	0.0
Res-R and B-50(6)	9.07	1.42	28.0	0.7

In almost all instances, the concentration of bound hapten determined in duplicate equilibrium dialysis experiments was within 5% of the mean value, and this deviation was taken to

FIGURE 14

BINDING CURVE OBTAINED BY EQUILIBRIUM DIALYSIS FOR THE REACTION OF R^o WITH THE ANTIBODIES IN B-50(5)

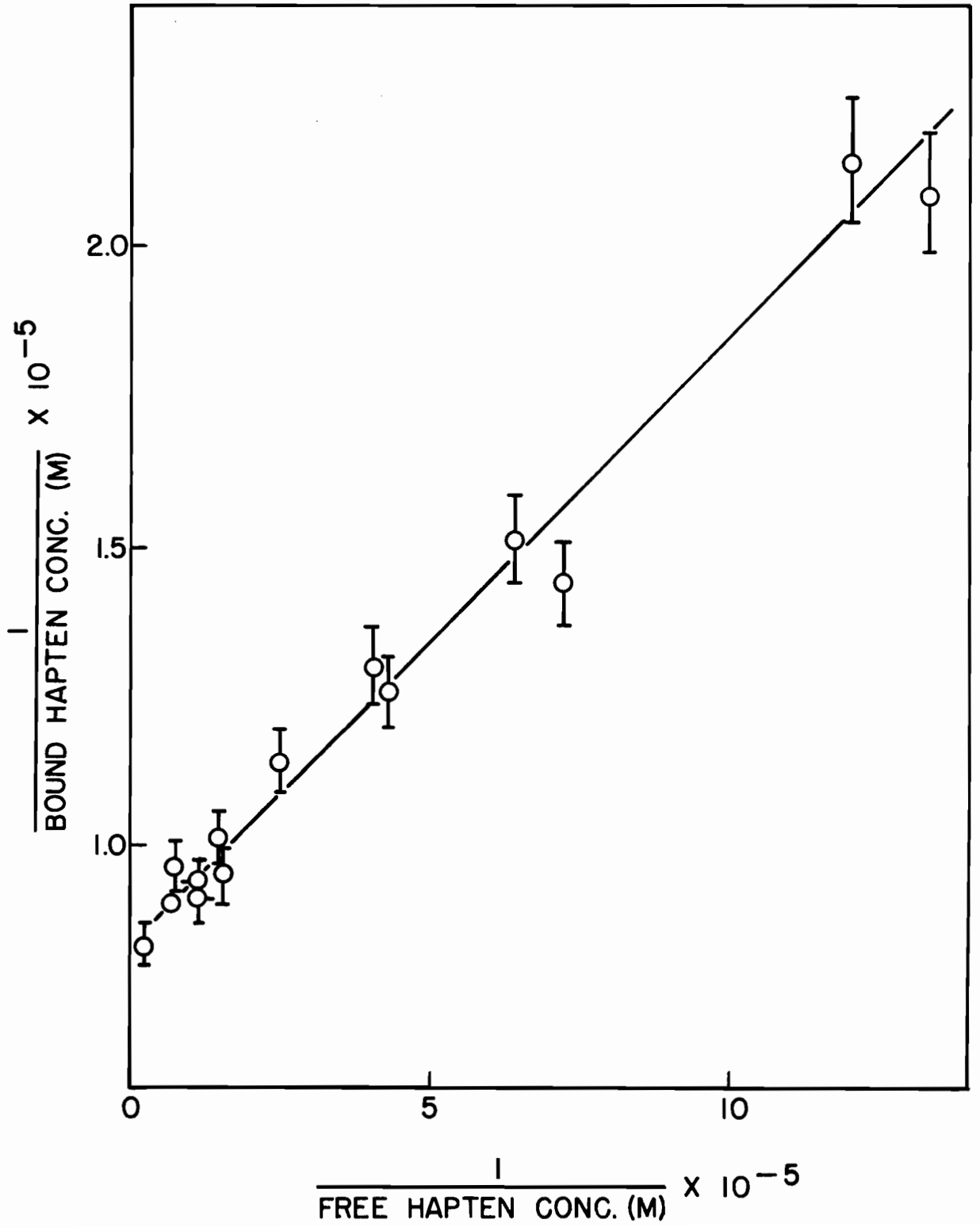
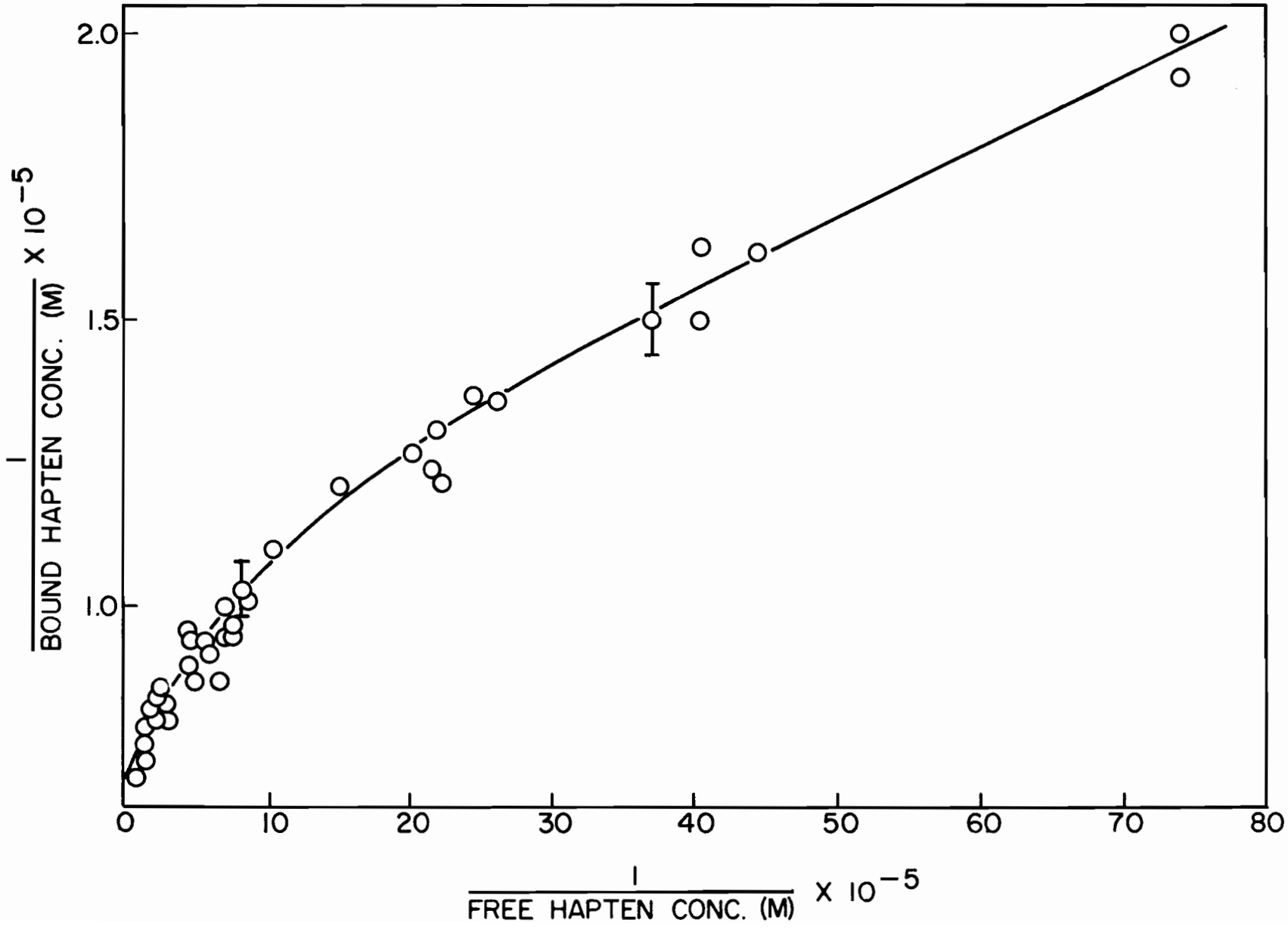


FIGURE 15

BINDING CURVE OBTAINED BY EQUILIBRIUM DIALYSIS
FOR THE REACTION OF RES-R WITH THE ANTIBODIES IN B-50(6)


2.0
1.5
1.0
0
10
20
30
40
50
60
70
80
1 / BOUND HAPTEN CONC. (M) X 10⁻⁵
1 / FREE HAPTEN CONC. (M) X 10⁻⁵

represent the probable error in the determination of the concentration of bound hapten. Within this error, the data in the binding curve for B-50(5) (Fig. 14) fall along a straight line in marked contrast to the curvature of the plot obtained with the preparation B-50(6) (Fig. 15). This behaviour is interpreted to mean that the antibodies in B-50(5) had very nearly the same affinity for R^o while the antibodies in B-50(6) exhibited a higher degree of heterogeneity for Res-R. The index of heterogeneity for this latter system was found to be 0.7 on the basis of the treatment of Sips (84) (Fig. 16 page 77), from which it can be deduced that 75% of the sites had an equilibrium constant in the range of $0.16K_o$ to $6K_o$ (84).

From plots of the number of hapten molecules bound non-specifically as a function of the number of free hapten molecules (Figs. 17 and 18) the amount of hapten bound non-specifically to the membrane and to normal gamma-globulins present in the antibody preparations was found to be 7 and 19% of the amount of free R^o and Res-R, respectively. The concentration of hapten bound only to normal gamma-globulins, H_n, was found to be equal to 2 and 9% of the free concentration of R^o and Res-R, respectively, from plots of H_n as a function of the free hapten concentration (Figs. 19 and 20).

The intrinsic equilibrium constants and amount of non-specific binding obtained for the systems investigated are

FIGURE 16

EVALUATION OF THE INDEX OF HETEROGENEITY
FOR THE BINDING OF RES-R WITH THE ANTIBODIES
IN B-50(6)

The method used to evalute the index of heterogeneity was that of Sips (page 77).

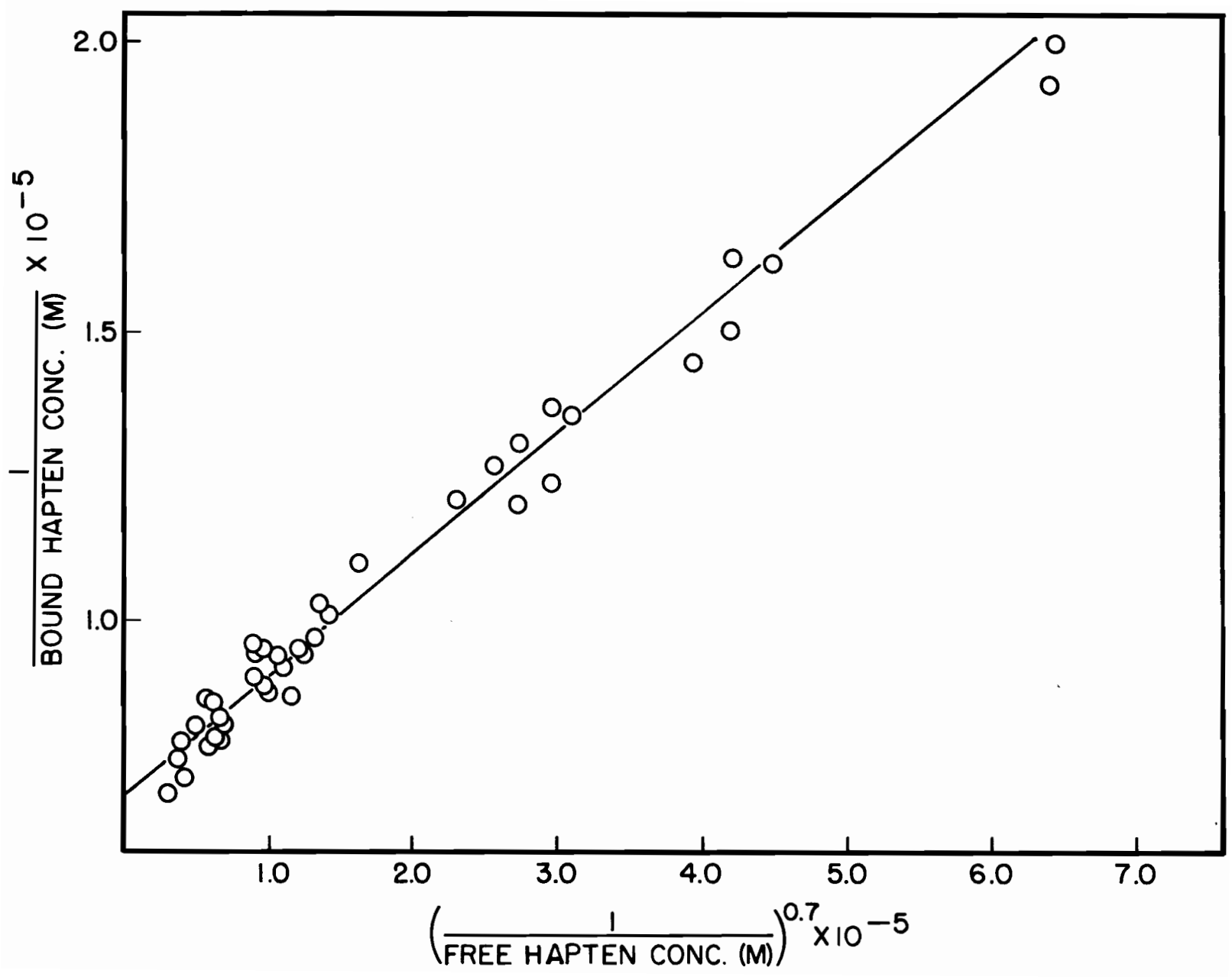

2.0
1.5
1.0
1 / BOUND HAPTEN CONC. (M) X 10^-5
1.0
2.0
3.0
4.0
5.0
6.0
7.0
(1 / FREE HAPTEN CONC. (M))^0.7 X 10^-5

FIGURE 17

AMOUNT OF R^{o} BOUND NON-SPECIFICALLY BY NORMAL RABBIT GAMMA-GLOBULIN AND BY THE DIALYSIS MEMBRANE

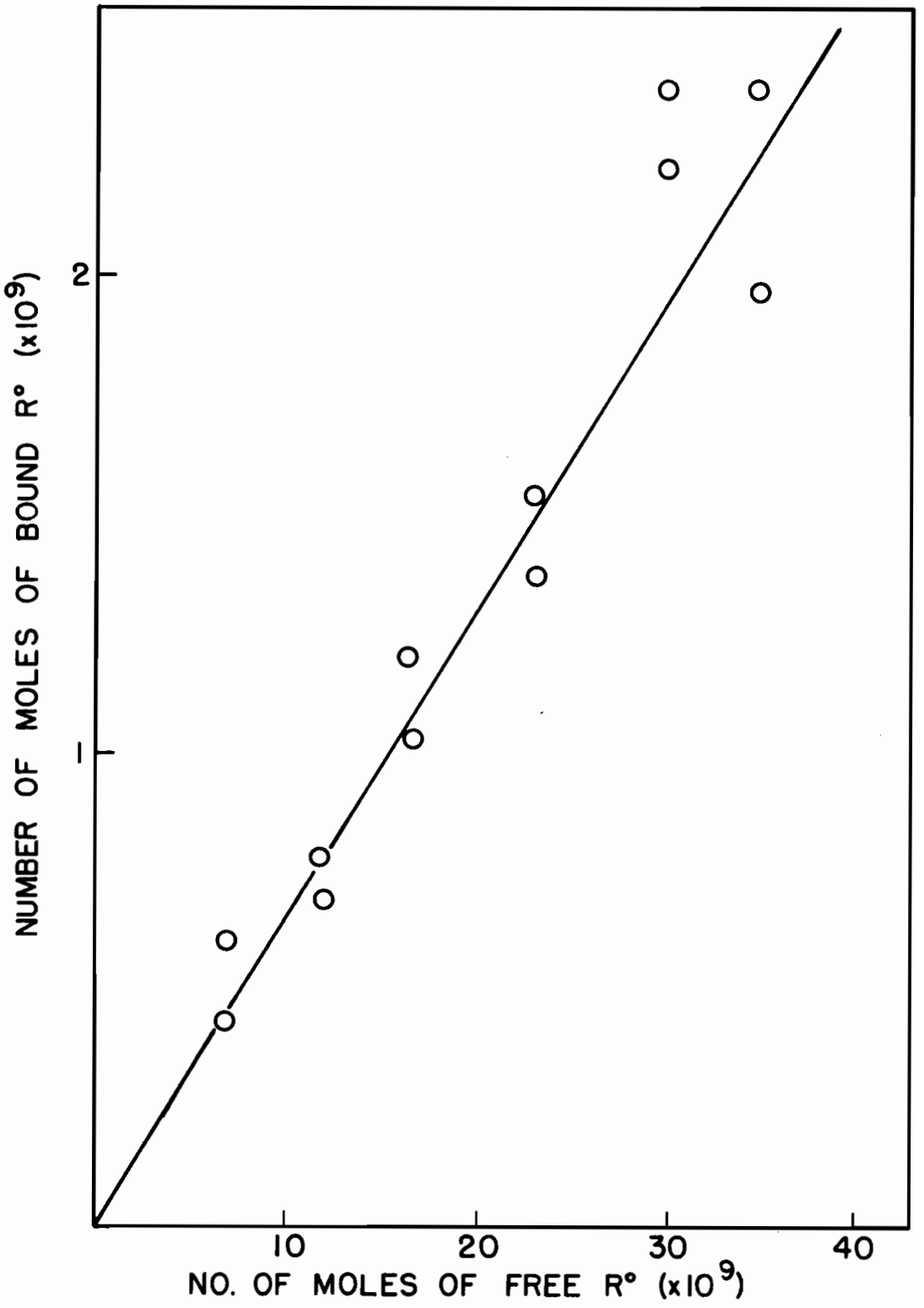


FIGURE 18

AMOUNT OF RES-R BOUND NON-SPECIFICALLY BY NORMAL RABBIT GAMMA-GLOBULIN AND BY THE DIALYSIS MEMBRANE

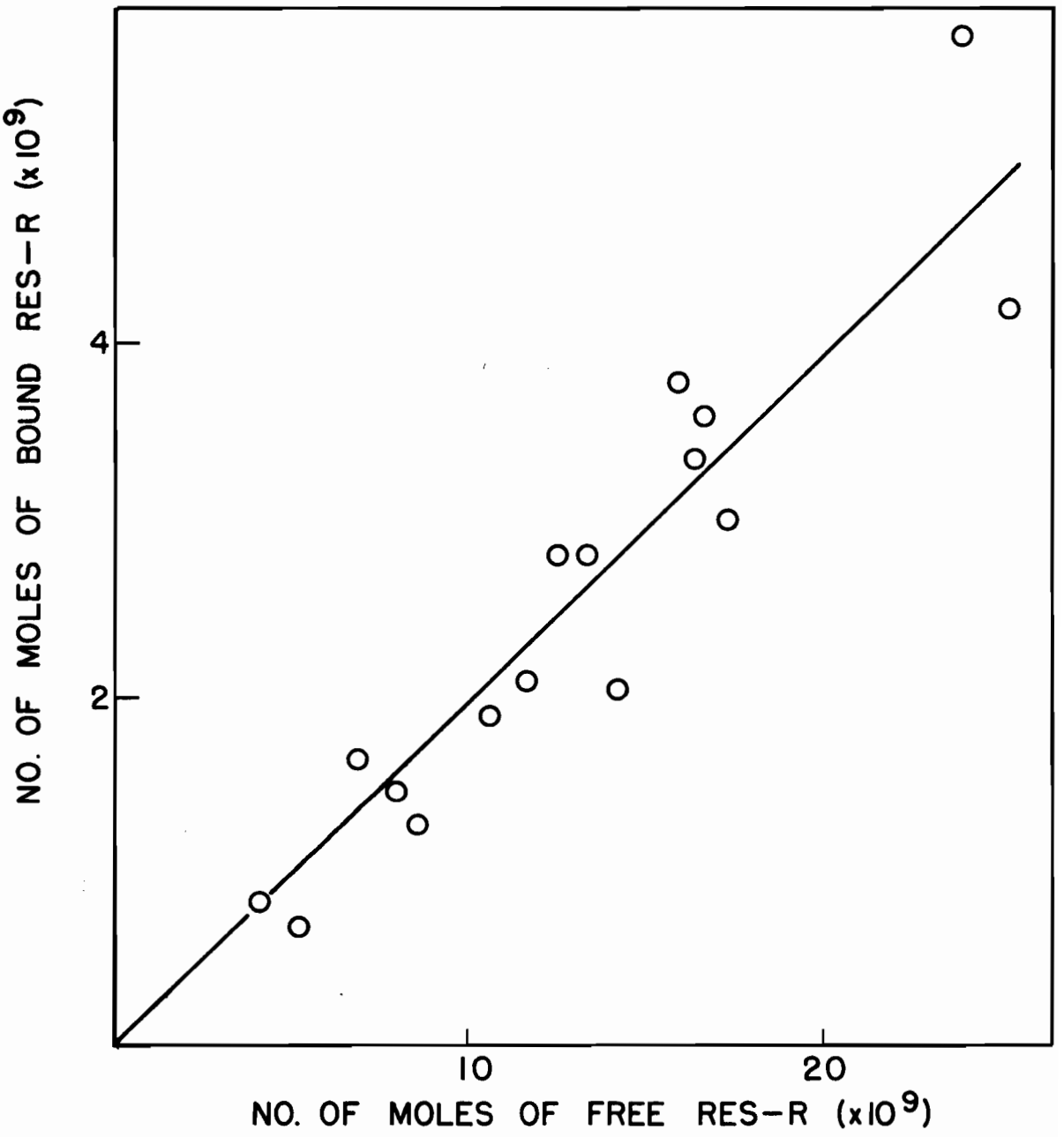


FIGURE 19

CONCENTRATION OF R^{o} BOUND BY

NORMAL RABBIT GAMMA-GLOBULIN

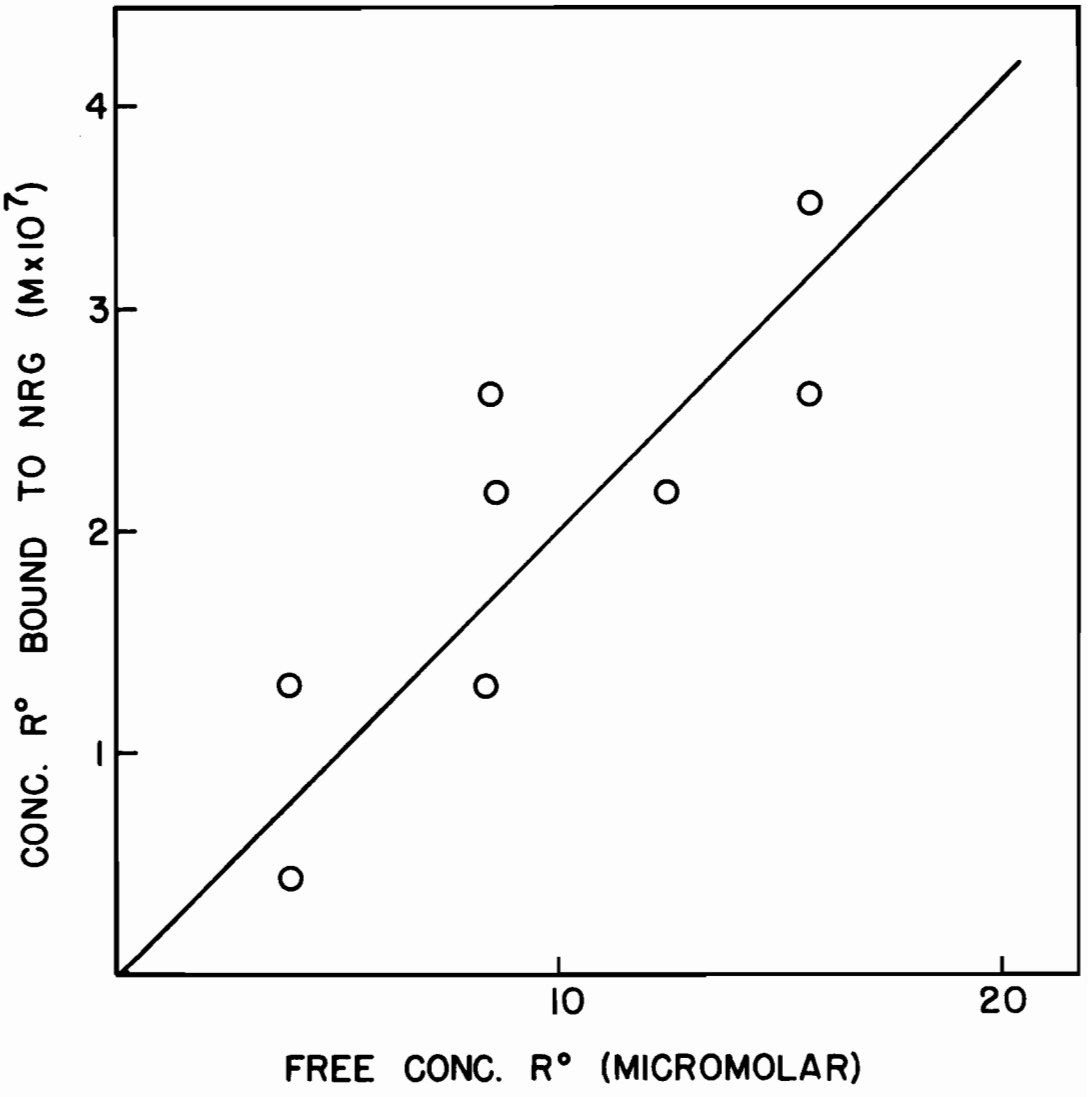
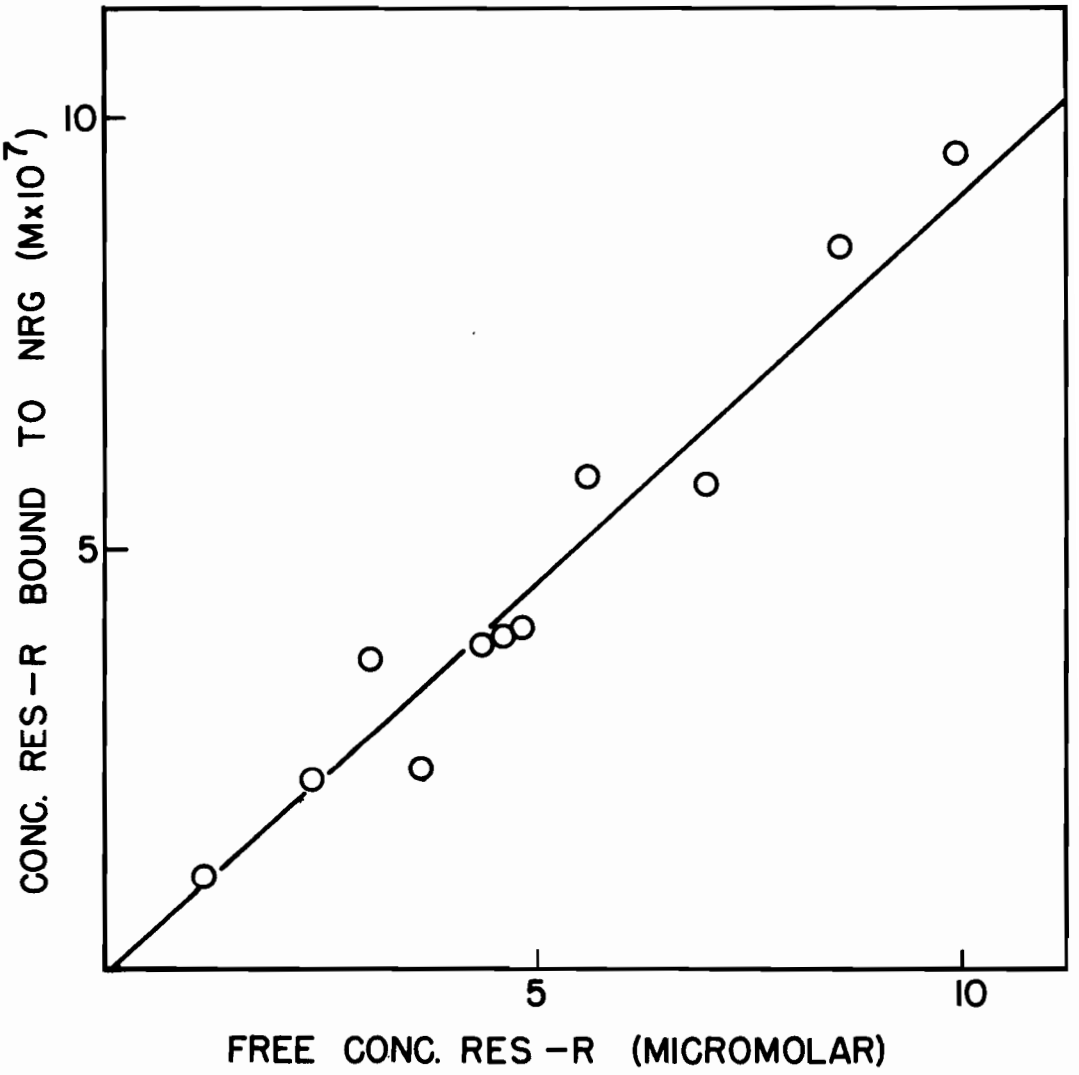


FIGURE 20

CONCENTRATION OF RES-R BOUND

BY NORMAL RABBIT GAMMA-GLOBULIN



of the same order of magnitude as those obtained by other workers with similar systems (31, 32, 84, 87). In addition, the index of heterogeneity for the binding of Res-R with the antibodies in B-50(6) is also similar (84). However, the apparent absence of heterogeneity in the reaction of R^o with the antibodies in B-50(5) is unusual, especially since the preparation B-50(5) is a gamma-globulin fraction isolated from pooled rabbit antisera, and Nisonoff and Pressman (84) have shown that the antibodies to a given hapten isolated from different rabbits combine with the hapten with different value for K_o. In view of these facts it is likely that there is heterogeneity in the reaction of R^o with B-50(5) but that it is not appreciable, and therefore, cannot be detected readily.

The factors which determine the amount of heterogeneity in a given antiserum are not known. The antisera comprising B-50(5) were obtained after only a single course of immunization, while those comprising B-50(6) were obtained from rabbits which had undergone at least one previous immunization. Therefore, it is possible that the length of time for which the animal is immunized determines the extent of heterogeneity. This view is supported by evidence obtained by other workers from precipitin curve experiments (13).

Part 2 - Polarographic Studies of Antibody - Hapten Reactions

The Effect of Rabbit Gamma-Globulins on Oscillographic Polarograms

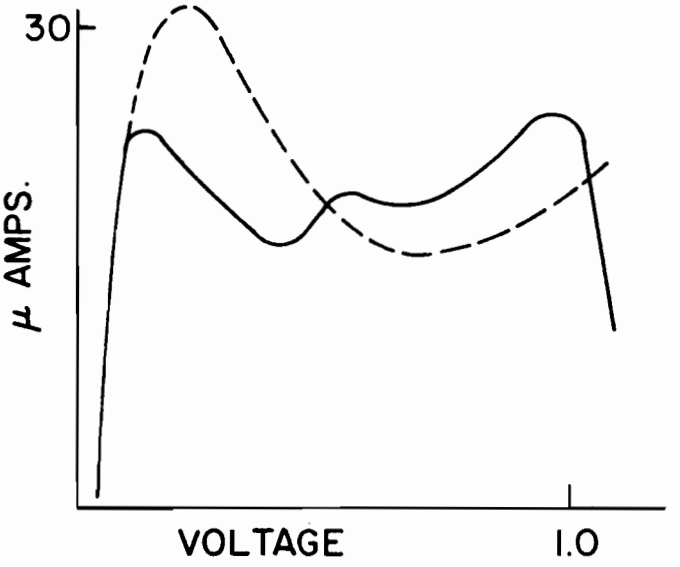
As illustrated in Fig. 21, the oscillographic polarograms obtained with the two antibody solutions, B-50(5) and B-50(6), were similar to those obtained with NRG solutions; however, they differed markedly from those obtained with solutions which did not contain protein. The polarograms obtained with the protein solutions exhibited a small peak at a potential of about -0.5 volts with respect to the saturated calomel electrode. This peak might have been due to (a) the reduction of some of the intramolecular disulphide bonds present in antibody and gamma-globulin molecules (since the disulphide bond of cystine, the amino acid containing this bond is reduced at a similar potential (155)), or (b) to a change in the capacity current of the DME in the presence of globulins. The origin of the peak was not investigated in detail, but it is likely that the observed changes in capacity current were caused by the adsorption of proteins on the dropping mercury electrode. This explanation is supported by the findings of other workers that proteins (138) and polypeptides (137) are adsorbed on mercury. It is supported also by the observation made in this study that the mercury drops falling from the electrode did not coalesce when the electrode was immersed in the gamma-globulin and

FIGURE 21

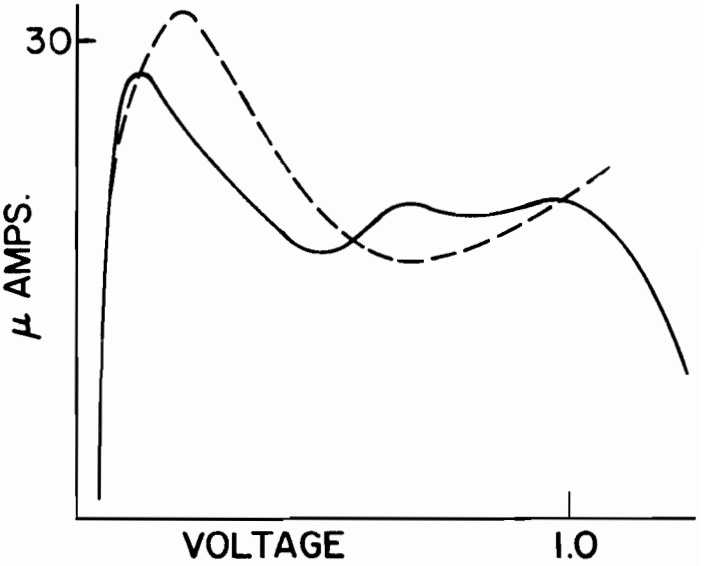
COMPARISON OF THE OSCILLOGRAPHIC POLAROGRAMS GIVEN BY THE BUFFER, ANTIBODY AND NORMAL RABBIT GAMMA-GLOBULIN SOLUTIONS

All voltages shown are referred to the saturated calomel electrode.

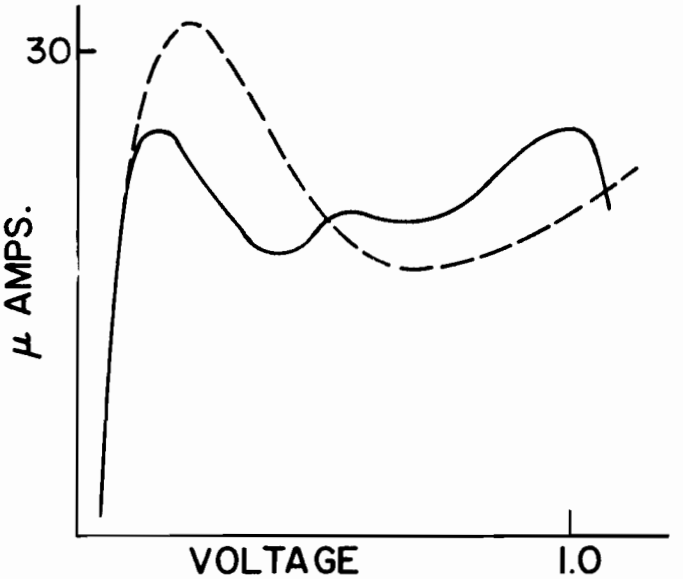
B-50(5) - SOLID LINE
BUFFER - DASHED LINE



B-50(6) - SOLID LINE
BUFFER - DASHED LINE



NRG - SOLID LINE
BUFFER - DASHED LINE



antibody solutions. As suggested by Tanford (136), this phenomenon probably results because the mercury drops become coated with a continuous layer of protein.

The Effect of Gamma Globulin on the Peak Height and Peak Potential of the Reducible Haptens

The peak height yielded by a given concentration of R^{o} and Res-R was lower in the presence of NRG than in the protein-free buffer. This effect is illustrated for R^{o} in Figs. 22 and 23. It can be seen from Fig. 22 that the peak heights obtained with solutions of the hapten R^{o} in the presence of 0.9% NRG, i.e. at very nearly the same protein concentration as used in the equilibrium dialysis experiments with the B-50(5) antibody preparation (1.028%), were approximately one-half of those obtained in the proteinfree buffer. Essentially similar results were obtained with the compound Res-R.

As was shown by equilibrium dialysis experiments, normal gamma-globulin solutions containing the same concentration of protein as the antibody solutions were capable of binding R^{o} and Res-R, but only to the extent of 2 and 9% of the concentration of the free form of each of these haptens, respectively. It is conceivable, therefore, that some of the lowering of the peak currents in the presence of globulins was due to the formation of non-reducible complexes. However, this effect would be expected to be rather small,

FIGURE 22

EFFECT OF NORMAL RABBIT GAMMA-GLOBULIN

ON THE PEAK HEIGHT GIVEN BY R°

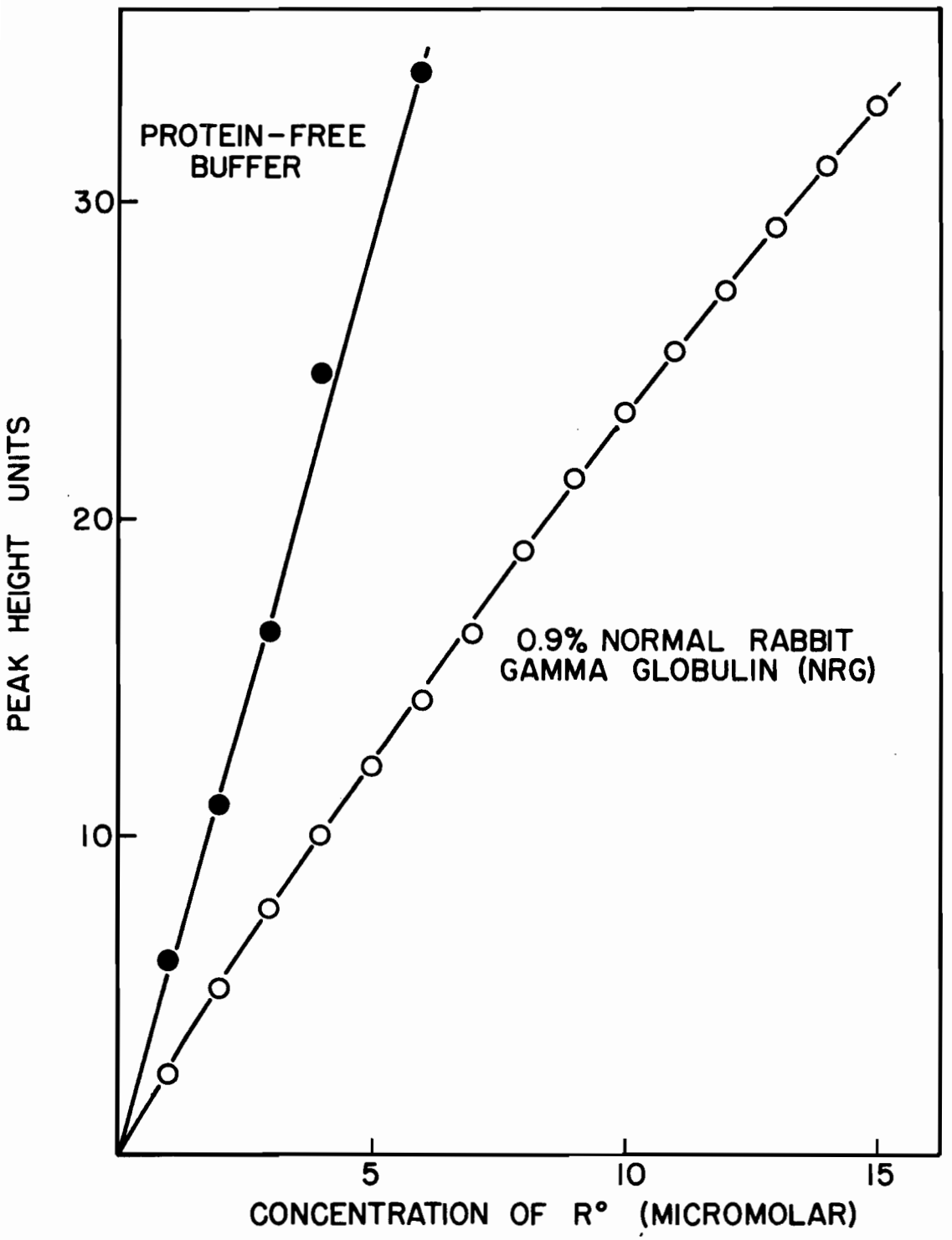
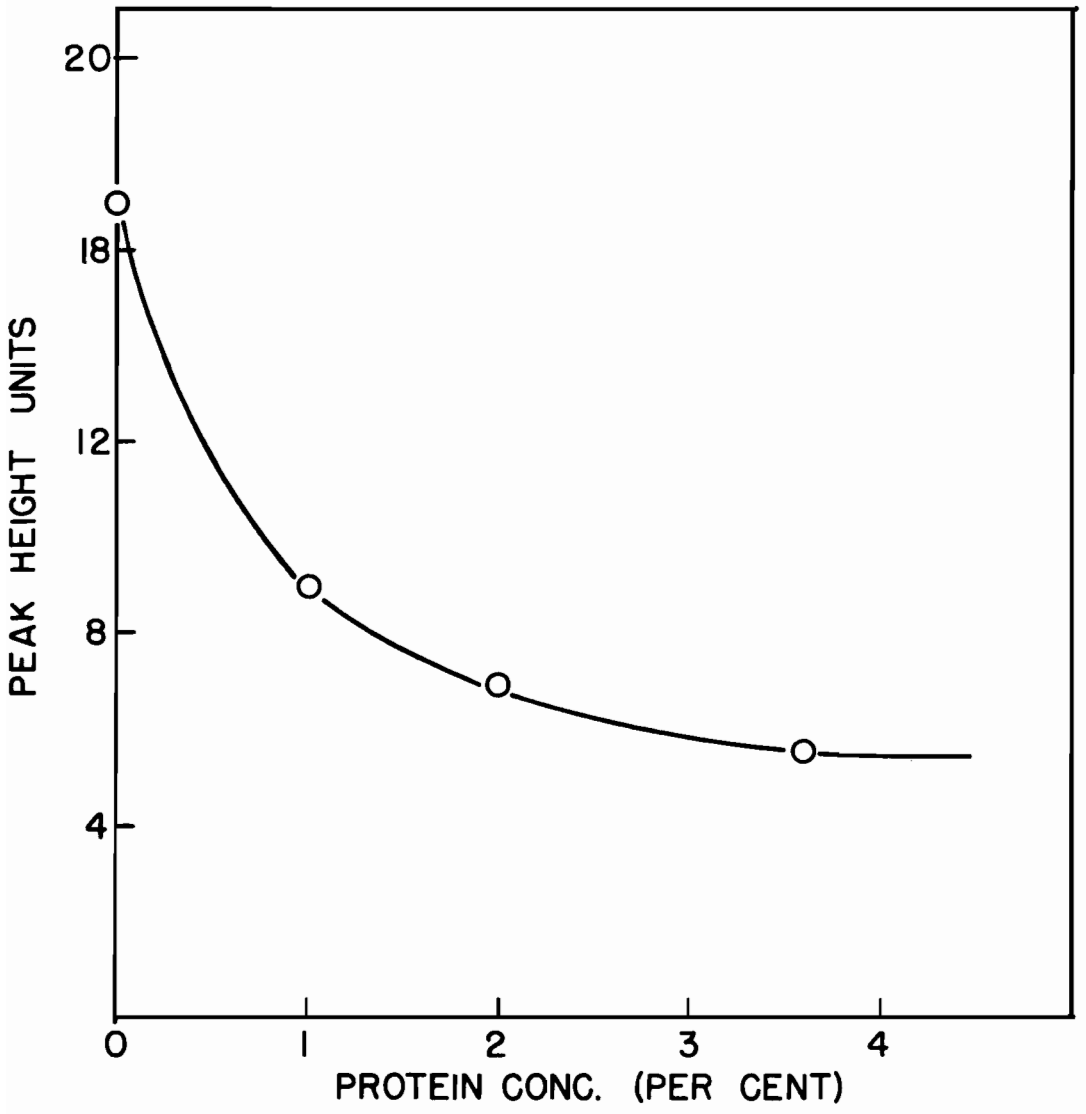


FIGURE 23

EFFECT OF THE CONCENTRATION OF NRG ON THE PEAK HEIGHT YIELDED BY A FIXED CONCENTRATION OF R^{O}

The data in this figure are for a R^{O} concentration of 3.5×10^{-6} molar.


20
18
12
8
4
PEAK HEIGHT UNITS
0
1
2
3
4
PROTEIN CONC. (PER CENT)

i.e., up to 9%, and not as large as the 50% decrease in the peak height observed. In view of the evidence presented above for the adsorption of normal rabbit globulins on the dropping mercury electrode (which would be expected to result in the lowering of the faradaic current yielded by the depolariser (page 37)), it is suggested that the decrease in peak heights was caused mainly by the adsorption of gamma-globulins.

In the absence of gamma-globulins, the peak potential for reduction of R^{O} and Res-R was determined as -0.45 and -0.55 volts, respectively, with respect to the saturated calomel electrode. However, in the presence of proteins these values were shifted by about 10 millivolts towards more negative values. Such shifts are in general associated with the adsorption of some material on the surface of the electrode (127) and, therefore, support further the view that gamma-globulin molecules were adsorbed on the mercury surface.

Since the physical properties of antibodies are indistinguishable from those of normal gamma globulins, it is probable that antibodies were adsorbed at the mercury surface in the same manner as the normal globulins.

The Measurement of Hapten Binding by Polarography

In the absence of any complicating effects, such as due to the adsorption of proteins on the dropping mercury elec-

trode or to the reduction of the bound form of the hapten, the amount of the reducible hapten bound specifically by the homologous antibodies would be measured by the decrease in the peak height with respect to the value obtained in the absence of antibodies. To eliminate the effects caused by the adsorption of protein on the DME, the following procedure was employed. A calibration curve was constructed for each protein solution by determining the peak heights for various hapten concentrations in the presence of a large excess (with respect to the antibody concentration) of benzenarsonic acid (BAA), which is structurally closely related to the reducible haptens. The peak heights yielded by identical hapten concentrations in antibody solutions in the absence of BAA were then measured. The observed decrease in peak height for a given hapten concentration, with respect to the corresponding value obtained from the calibration curve, was considered to be due to the specific binding of the hapten by the antibody. Typical curves obtained in this manner with the system consisting of R^o and B-50(5) are shown in Fig. 24. The rationale for using this procedure was that the extent of the reaction between the reducible hapten and the antibody, i.e. reaction (1).

$$Ab + R^o \rightleftharpoons Ab - R^o \qquad (1)$$

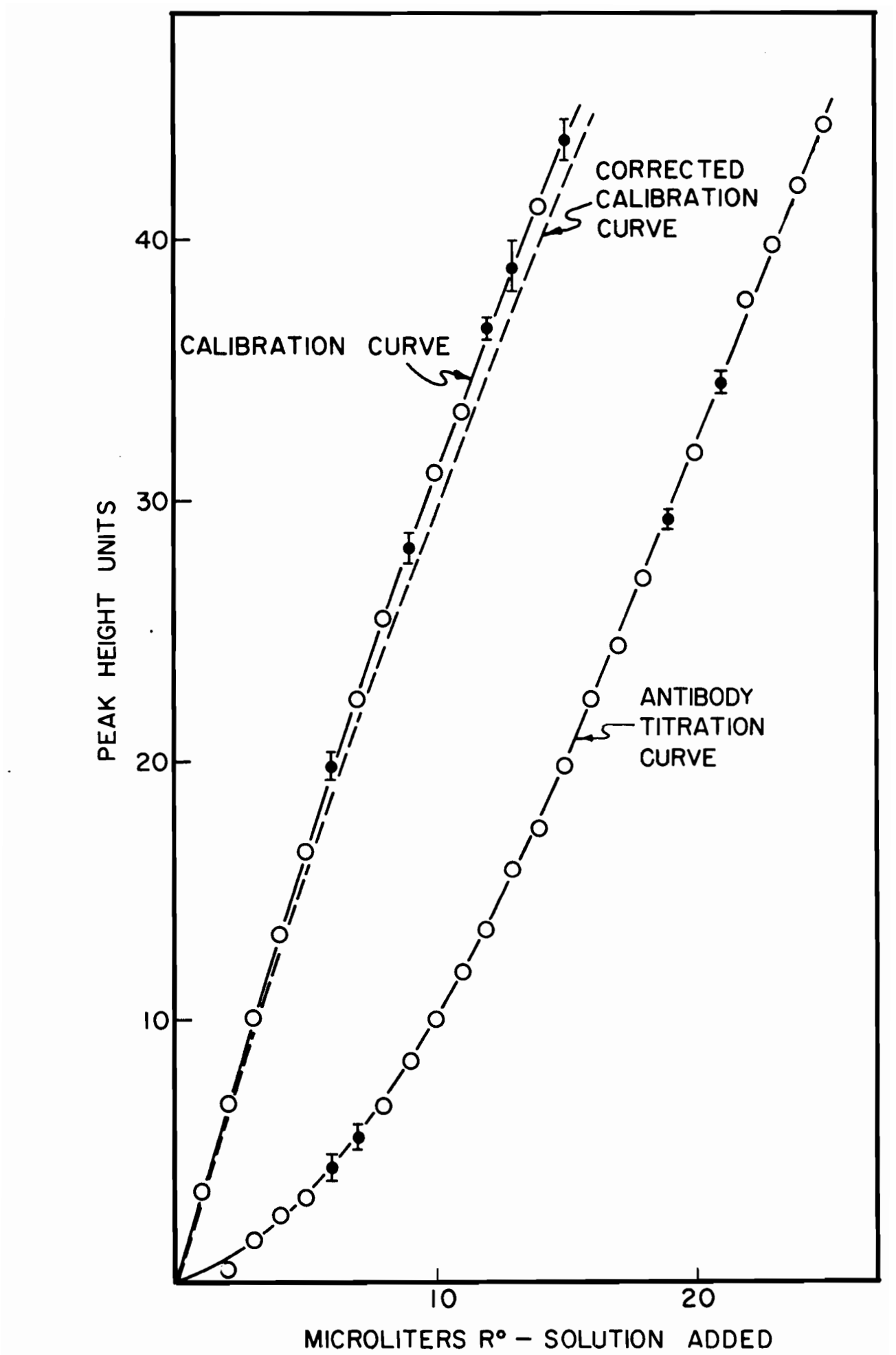
would be negligible compared to reaction (2),

$$Ab + BAA \rightleftharpoons Ab - BAA \qquad (2)$$

FIGURE 24

CALIBRATION AND ANTIBODY TITRATION CURVES
FOR THE SYSTEM CONSISTING OF R^o AND B-50(5)

The antibody titration curve represents the peak height measured after the addition of various amounts of the reducible hapten R^o to the antibody solution. The calibration curve represents the peak heights measured when R^o was added to the antibody solution in the presence of a large excess (10^{-3} molar) of the non-reducible inhibitor benzenearsonic acid (BAA). The corrected calibration curve was derived from the calibration curve by the application of a correction factor to account for the increase in peak height caused by BAA in solutions of gamma-globulins (see text, page 96). The volumes of solution used to obtain the calibration and antibody titration curves were 1.04 ml. The concentration of the R^o solution added was 1.04×10^{-3} molar.


CORRECTED
CALIBRATION
CURVE
CALIBRATION CURVE
ANTIBODY
TITRATION
CURVE
PEAK HEIGHT UNITS
40
30
20
10
10
20
MICROLITERS R° – SOLUTION ADDED

in the presence of a large excess of BAA. In actual fact, from equilibrium dialysis experiments it was found that the amount of reducible hapten bound specifically in the presence of 2000-fold molar excess of BAA (with respect to antibody concentration) was not more than 2% of the total amount of hapten present in solution. Therefore, this procedure was considered to be appropriate, and for all quantitative experiments calibration curves were obtained in the presence of a 2000-fold excess of BAA (corresponding to 10^{-3} Molar).

The effect of BAA on the peak height in solutions of normal gamma-globulins containing the same protein concentration as the antibody solutions used in the polarographic experiments was also investigated. As is evident from Figs. 25 and 26, the peak heights in the presence of BAA were slightly higher, this effect being of the order of 4 and 6% for R^{o} and Res-R, respectively. In view of the rather small magnitude of this effect no attempt was made to elucidate the mechanism underlying it. However, it is suggested that this increase could be due to one or a combination of several factors, such as the inhibition of non-specific binding by BAA, a change in electrical properties of the solution, or an alteration of the structure of the electrical double layer at the mercury-solution interface. Since both B-50(5) and B-50(6) consisted mainly of normal gamma-globulins (Table II), the calibration curves were corrected accordingly for the effect of BAA as indicated by the corrected calibration curve

FIGURE 25

EFFECT OF BENZENEARSONIC ACID ON THE PEAK HEIGHT GIVEN BY R^{o} IN SOLUTIONS OF NORMAL RABBIT GAMMA-GLOBULINS

Protein and benzenearsonic acid concentrations were 9.04 mg/ml and 10^{-3} molar, respectively. The titrant, 1.04×10^{-3} molar R^{o}, was added to 1.04 mls of protein solution.

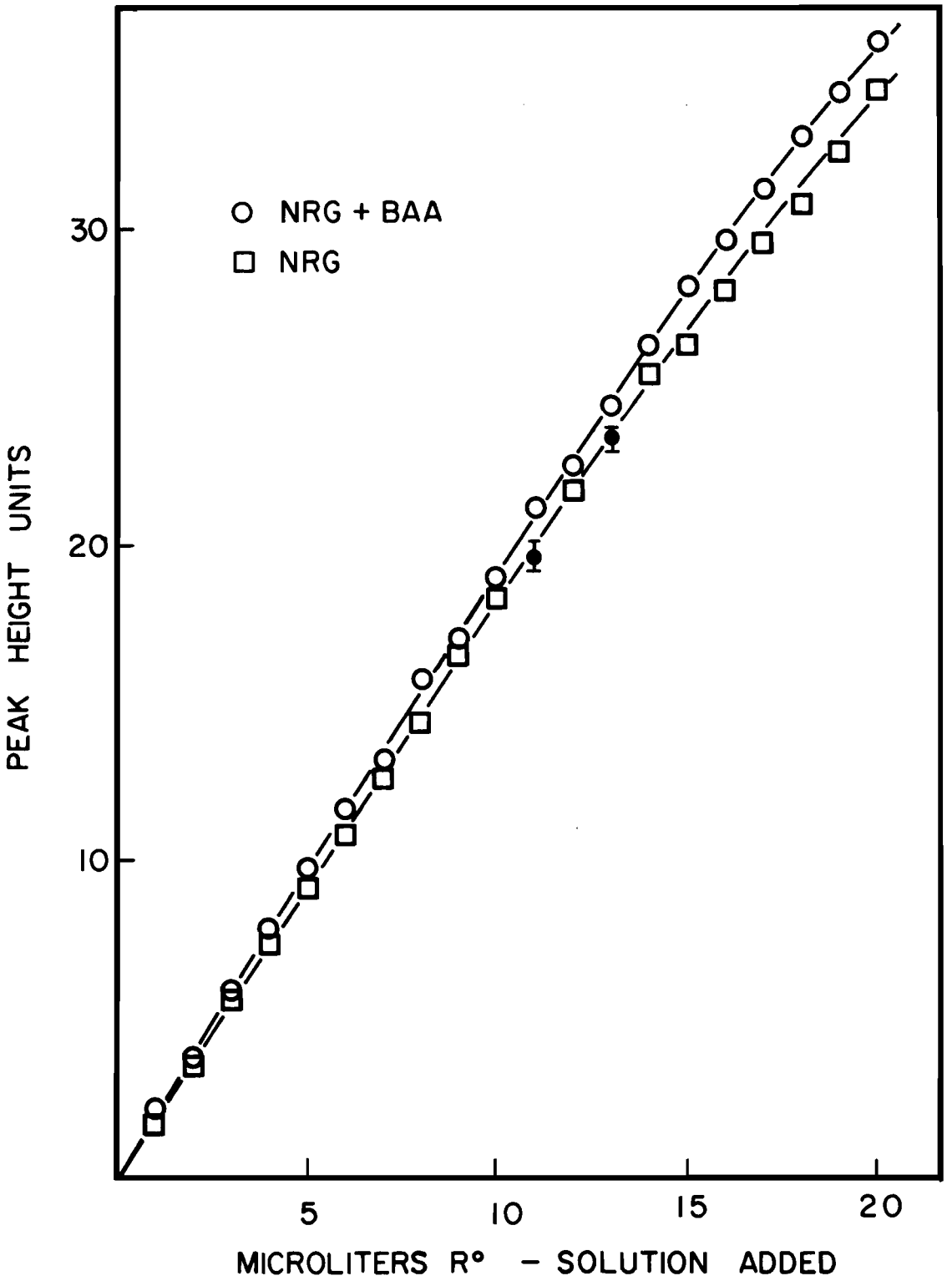
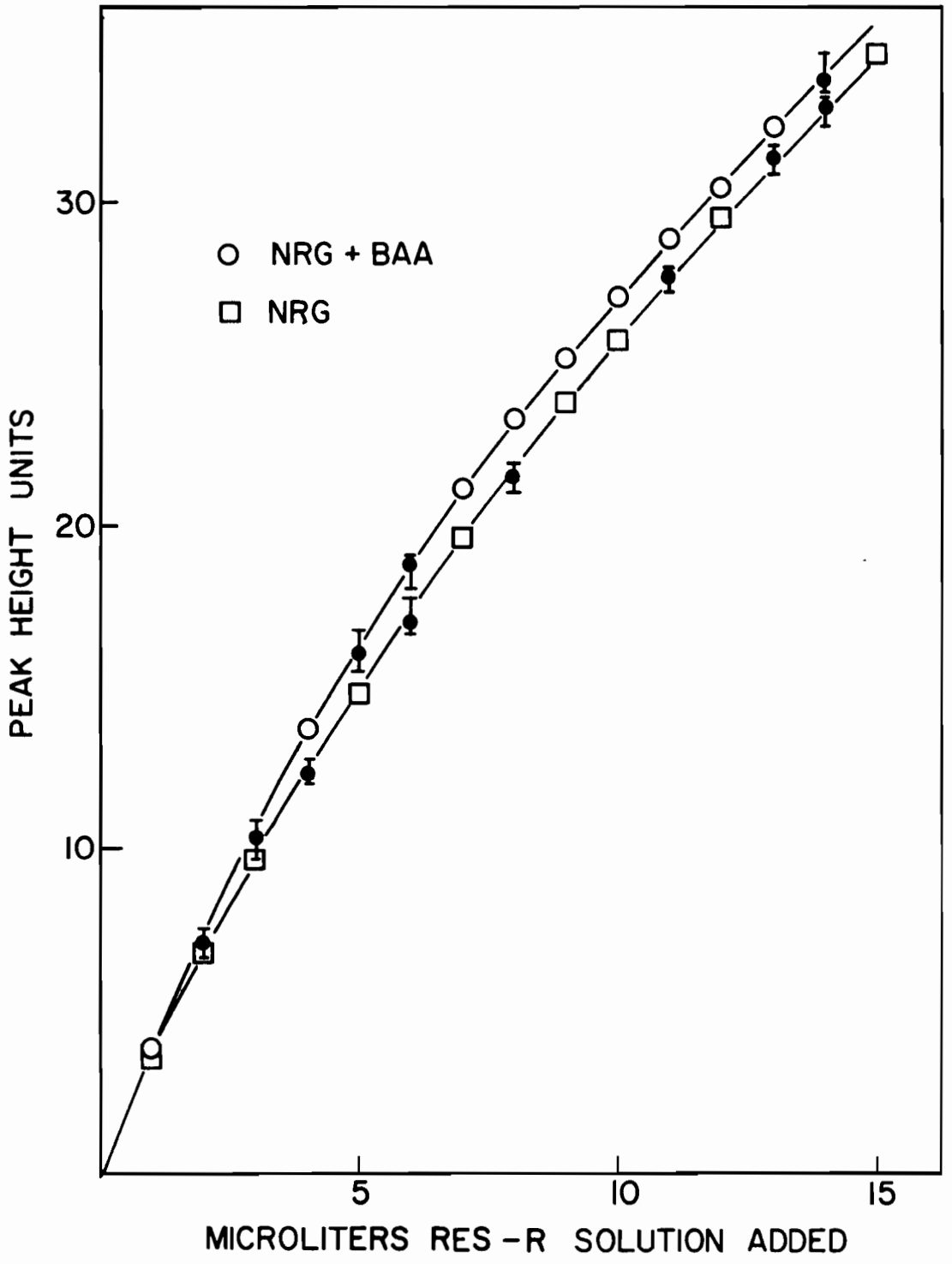

NRG + BAA
NRG
30
20
10
PEAK HEIGHT UNITS
5
10
15
20
MICROLITERS R° – SOLUTION ADDED

FIGURE 26

EFFECT OF BENZENEARSONIC ACID ON
THE PEAK HEIGHT GIVEN BY RES-R IN
SOLUTIONS OF NORMAL RABBIT GAMMA-GLOBULINS

Protein and benzenearsonic acid concentrations were 9.48 mg/ml. and 10^{-3} molar, respectively. The titrant, 1.04 x 10^{-3} molar Res-R, was added to 1.04 mls of protein solution.


30
20
10
PEAK HEIGHT UNITS
O NRG + BAA
□ NRG
5
10
15
MICROLITERS RES-R SOLUTION ADDED

in Fig. 24.

It is readily evident that if the faradaic currents measured when the antibody titration curve was obtained were due only to the reduction of free hapten, then this curve could be considered to represent a plot of the free hapten concentration as a function of the total concentration of hapten in the antibody solution. Similarly, the corrected calibration curve could be considered to represent a plot of the free hapten concentration versus the total hapten concentration for the antibody solution under conditions where specific binding was eliminated. It is interesting to note in this connection the striking similarity between the corrected calibration curve, the antibody titration curve (Fig. 24), and theoretical plots for the free hapten concentration as a function of the total hapten concentration (Fig. 27) computed for several values of the intrinsic equilibrium constant, Kint.

The details of the procedure employed in calculating the concentration of the free and bound hapten from the corrected calibration and antibody titration curves are illustrated in Fig. 28. The peak height a, obtained with the antibody solution, was considered to be identical to the peak height a' yielded by a solution of hapten in the presence of BAA, as given by the corrected calibration curve. Hence, the concentration of free hapten in the presence of homologous antibodies was taken to be given by

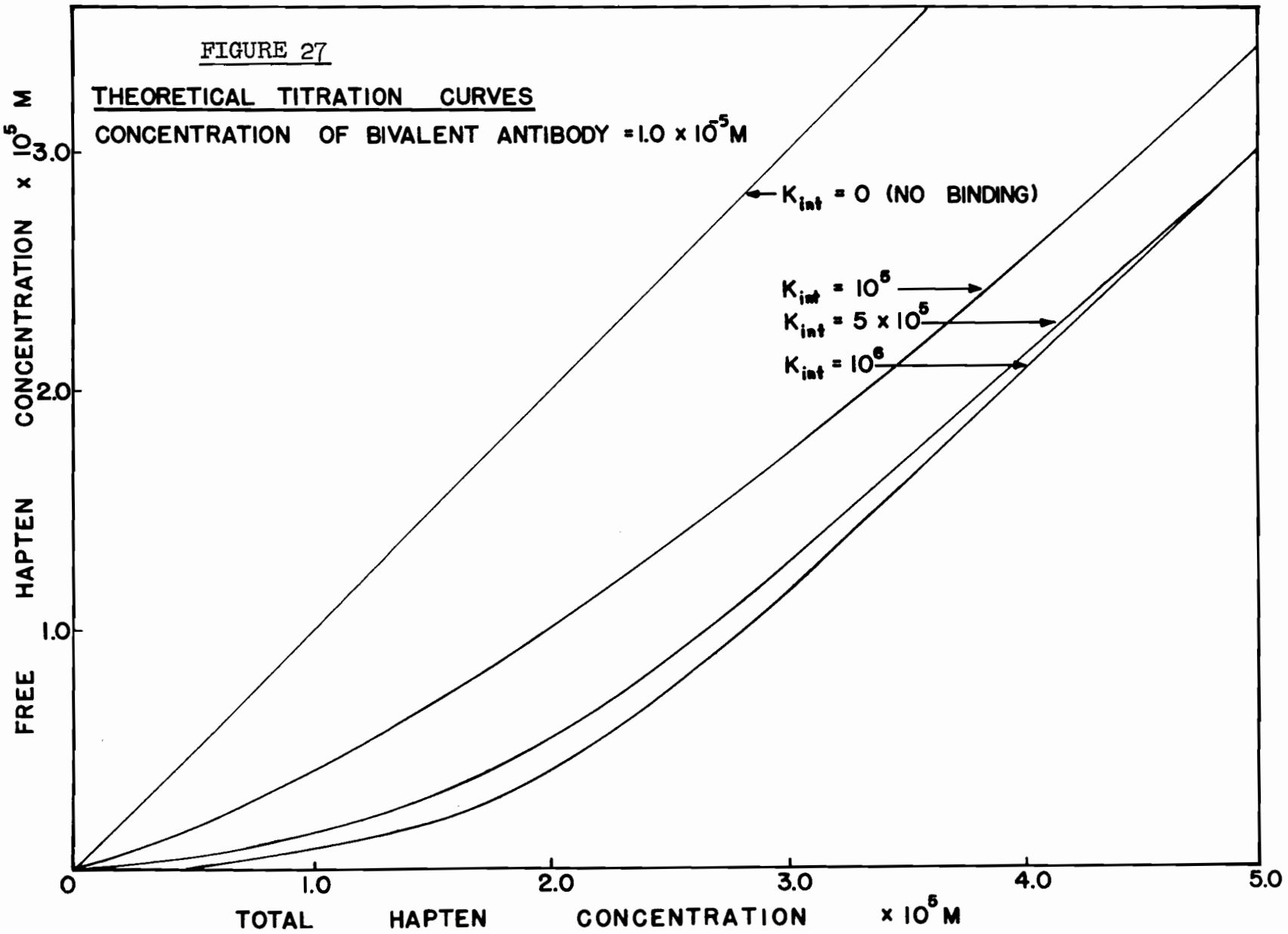
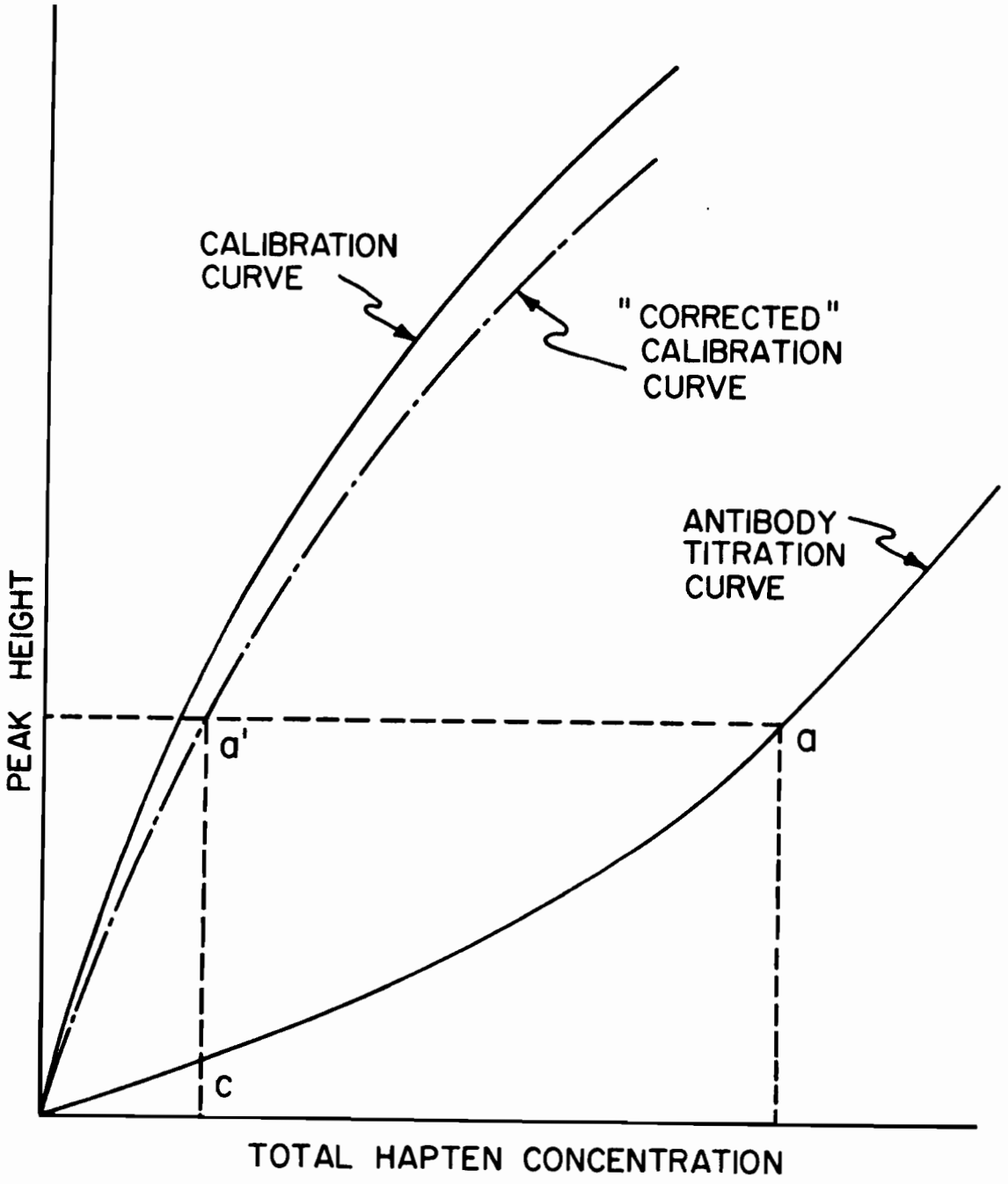


FIGURE 27
THEORETICAL TITRATION CURVES
CONCENTRATION OF BIVALENT ANTIBODY = 1.0 x 10⁻⁵M
FREE HAPTEN CONCENTRATION x 10⁵ M
3.0
2.0
1.0
0
1.0
2.0
3.0
4.0
5.0
TOTAL HAPTEN CONCENTRATION x 10⁵ M
K_int = 0 (NO BINDING)
K_int = 10⁵
K_int = 5 x 10⁵
K_int = 10⁶

FIGURE 28

ILLUSTRATION OF THE PROCEDURE USED TO COMPUTE THE FREE AND BOUND HAPTEN CONCENTRATIONS FROM POLAROGRAPHIC CALIBRATION AND ANTIBODY TITRATION CURVES



abscissa c. The concentration of bound hapten was then computed using the relation

$$b = (H_t) - H_n - c$$

where (H_t) represents the total concentration of hapten, and H_n, the concentration of that bound non-specifically by normal gamma-globulins. The values of H_n employed were obtained by means of equilibrium dialysis experiments, and as has been shown previously, (page 81) the amount of hapten bound non-specifically amounted only to 2 and 9% of the concentration of the free forms of the two haptens used. It is obvious that this calculation is valid provided that neither the hapten bound by antibody or by normal gamma-globulins is reducible at the same potential as the free hapten.

It is to be pointed out that for the determination of the calibration and antibody titration curves identical volumes and protein concentrations were employed. For the antibody titration curves 1.00 ml. of the antibody solution was used and 40 microliters of the buffer was added; correspondingly, for the calibration curves, 40 microliters of a solution of 0.5 molar BAA* was added to 1.00 ml. of the antibody solution.

Reproducibility of Polarographic Measurements

In general, all calibration and antibody titration curves

---

* This solution was prepared by dissolving BAA in the borate buffer and thenadjusting the pH to 8.0; this adjustment was required since the amount of BAA was sufficiently large to neutralize all of the available base in the buffer solution.

were determined at least in duplicate. The peak height in the different experiments was usually reproducible to within ±0.5 peak height unit. Hence, the average value of a peak height was represented in the various figures (Figs. 24, 25, 26) as a point in the center of a circle with a radius corresponding to 0.5 peak height units. Occasionally, larger deviations were observed; in such instances the average value was indicated in the plot together with the lowest and highest values measured.

Hapten Binding Curves Determined by Polarography

Binding curves obtained using the method described above in three out of five independent experiments with the system consisting of R^o and B-50(5) are shown in Fig. 29. The points obtained in the two experiments which are not shown lay within the lines obtained in the other three. The binding curve obtained in a typical experiment with Res-R and B-50(6) is shown in Fig. 30. For both systems the binding curves were practically straight lines; consequently, it was considered that the data could be treated as if these antibodies preparation had no appreciable heterogeneity with respect to the binding constants. The intrinsic equilibrium constants and antibody concentration were computed using the relation (page 77)

$$\frac{1}{b} = \frac{1}{c}\,\frac{1}{(KAb)} + \frac{1}{Ab}$$

FIGURE 29

BINDING CURVES OBTAINED POLAROGRAPHICALLY FOR THE REACTION OF R^{o} WITH THE ANTIBODIES IN B-50(5)

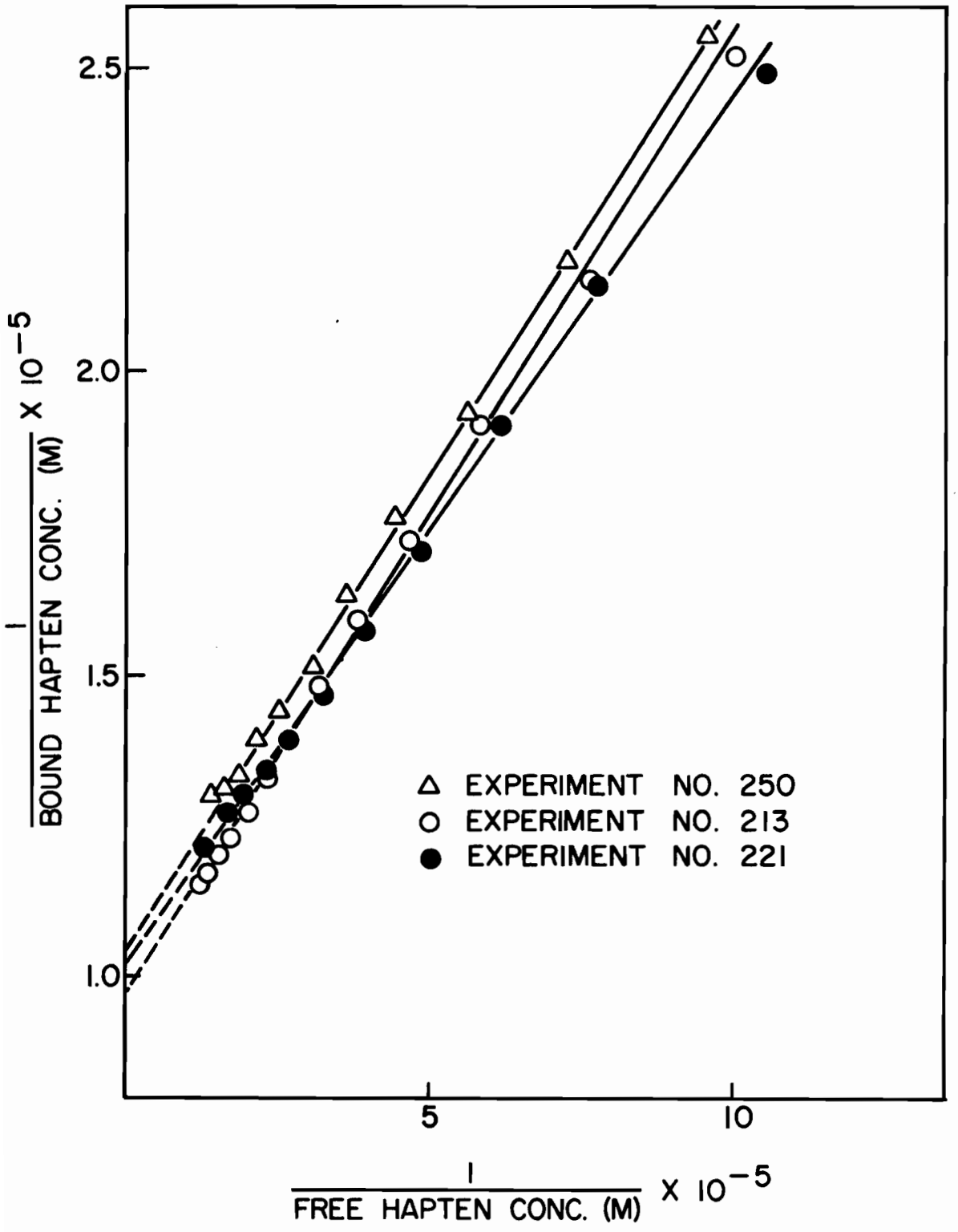
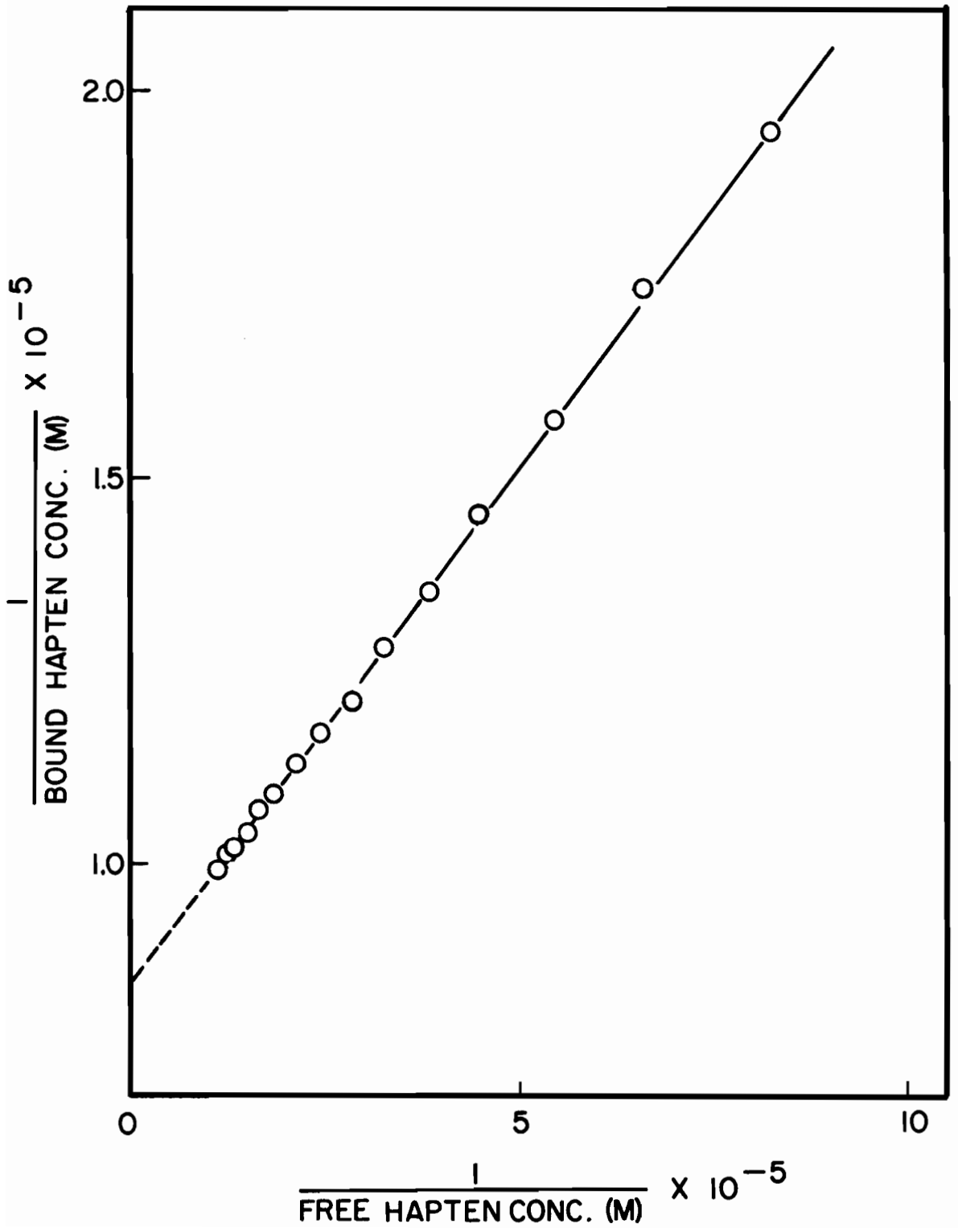


FIGURE 30

BINDING CURVES OBTAINED POLAROGRAPHICALLY FOR THE REACTION OF RES-R WITH THE ANTIBODIES IN B-50(6)



The values calculated for these parameters and other relevant data are summarized in Table IV.

TABLE IV

Intrinsic Equilibrium Constants Determined by Polarography

System	Expt.No.	K x 10^{-5} (1/mole)	Ab x 10^{5} (mole/l)	Prot.Conc. (mg/ml)
R° and B-50(5)	200	6.60	0.99	9.66
	206	6.95	1.00	9.69
	221	7.40	1.01	9.75
	213	6.05	1.08	9.69
	196	6.30	1.04	9.71
	Mean	6.65	1.02	9.70
Res-R and B-50(6)		6.40	1.17	9.48

Comparison of Results Obtained by Polarography and Equilibrium Dialysis

The results obtained by polarography and by equilibrium dialysis are compared in Table V.

TABLE V

Comparison of Binding Data Obtained by Polarography and by Equilibrium Dialysis

System	Method	Intrinsic Eqlm. Const. (1/Mole) x 10^{-5}	Index of Heterogeneity a
R° and B-50(5)	Eq.Dialysis	8.30	0.0
	Polarography	6.65	0.0
Res-R and B-50(6)	Eq.Dialysis	28.0	0.7
	Polarography	6.40	0.0

Although the intrinsic equilibrium constants determined by the two methods were of the same order of magnitude, the values obtained by polarography were somewhat smaller. This difference was significant, the ratio of the equilibrium constant amounting to 1.3 and 4.5 in the R^{o} - B-50(5) and Res-R - B-50(6) systems, respectively. Moreover, the polarographic data did not show any appreciable degree of heterogeneity in the Res-R system. Since the results obtained by equilibrium dialysis are usually considered to be reliable, it is concluded that the discrepancy is the result of some feature peculiar to the polarographic method.

The manner in which the concentration of hapten bound to antibody varies with the total hapten concentration (that is with the sum of the concentration of the free hapten and the concentration of the hapten bound by antibodies), as measured by polarography and by equilibrium dialysis is shown in Fig. 31 and 32. The data for the curves labelled equilibrium dialysis were derived using the value for the intrinsic equilibrium constants obtained in the equilibrium dialysis experiments, and the antibody concentration used in these calculations were those actually employed in the polarographic experiments. In this manner, small differences in the antibody concentrations used in the two types of experiments were compensated for (see Tables II and IV).

From a comparison of the curves in Figs. 31 and 32 it was found that the apparent concentration of bound hapten determined by

FIGURE 31

COMPARISON OF BINDING DATA OBTAINED BY POLAROGRAPHY AND BY EQUILIBRIUM DIALYSIS FOR THE SYSTEM CONSISTING OF R° AND B-50(5)

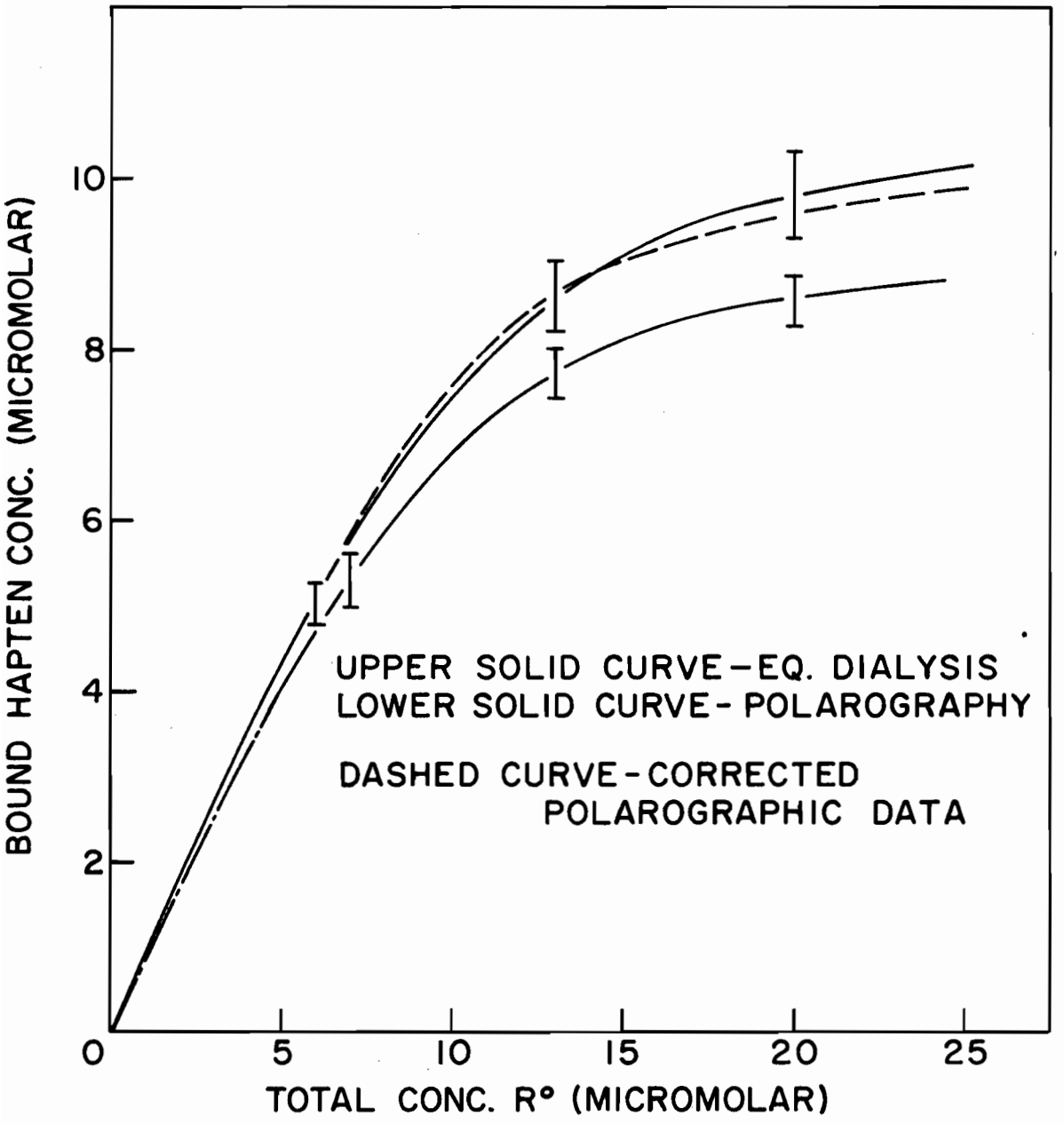
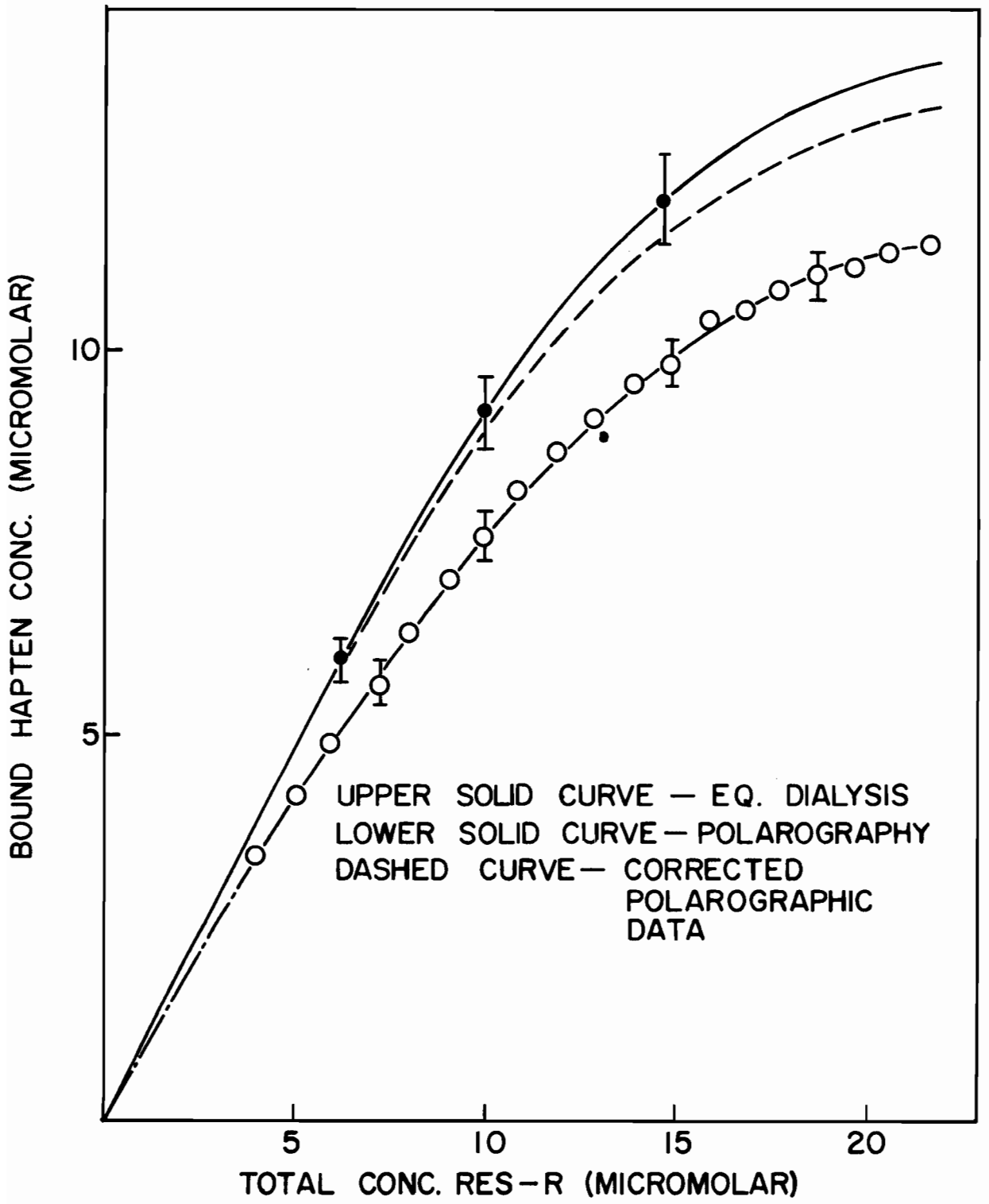


FIGURE 32

COMPARISON OF BINDING DATA OBTAINED BY POLAROGRAPHY AND BY EQUILIBRIUM DIALYSIS FOR THE SYSTEM CONSISTING OF RES-R AND B-50(6)



polarography was lower than that measured by equilibrium dialysis by about 10% and 17% for the systems consisting of antibodies and the haptens R^o and Res-R, respectively. In addition, the data indicate that the difference between the concentration of bound hapten measured by equilibrium dialysis and that measured by polarography increases at the higher concentration of hapten. Therefore, as a first approximation, it is suggested that this difference, Δb, is proportional to the concentration of bound hapten measured by equilibrium dialysis and which is denoted by b, the concentration of bound hapten as measured by polarography being denoted by b'. One can then write

$$\begin{aligned} \Delta b &= b - b' \\ &= Gb \end{aligned} \qquad (1)$$

where G is a proportionality constant. Equation (1) may be written as

$$b = \frac{b'}{1-G}$$

suggesting that if G were determined, the polarographic data could be corrected to yield binding data equivalent to those obtained by equilibrium dialysis. This was actually found to be the case as indicated by the curves for the corrected polarographic data shown in Figs. 31 and 32 which were computed by taking G to be equal to .091 and .145 for the R^o and Res-R systems, respectively. It is thus evident that the polarographic data can be corrected to yield binding

data equivalent to those obtained by equilibrium dialysis, provided the empirical correction factor G is evaluated from paralle equilibrium dialysis and polarographic experiments.

In spite of the obvious limitations of the polarographic procedure, it is evident that it may yield valuable data for the binding of haptens to antibodies, particularly in cases where other methods cannot be used easily.

Since the magnitude of the difference Δb seems to be a function of the concentration of bound hapten, b, it is suggested that it is due to the reduction of bound hapten, i.e., to the reduction of hapten in antibody-hapten complexes at the dropping mercury electrode at a potential indistinguishable from that of the free hapten. In considering the current yielded by such complexes it is possible to visualize at least two situations: (i) that every complex has the same intrinsic ability to yield a faradaic current at the DME, and (ii) that although all complexes can yield a faradaic current, the ease with which this can be done varies amongst the different complexes. From an inspection of the data in Table V and in Figures 31 and 32, it is obvious that the descrepancy is larger in the system consisting of Res-R and the antibody preparation B-50(6), which has more pronounced heterogeneity than the other system studied. This fact lends support to the second proposition. Thus, the equilibrium dialysis procedure would be expected to measure the binding of haptens to antibodies with both low and high affinities, whereas in

the polarographic procedure, the more weakly bound hapten molecules would be expected to be more readily reduced in the corresponding complexes or to become dissociated more easily from such complexes, the released hapten being then subsequently reduced.

The validity of the above explanation could be tested if the manner in which Δb varies with b were known with precision. In effect, G is not to be regarded as a true constant since it depends on the contribution of the reducible, bound hapten to the measured polarographic current. The ratio of the concentration of the weakly bound to that of the strongly bound hapten would vary with b since (1) at low concentrations of hapten (relative to the antibody concentration) one would expect that antibodies with highest affinity would react preferentially to form difficultly reducible complexes, and (2) at the higher hapten concentrations antibodies with lower affinities would also combine to form complexes which are readily reduced. Because of experimental errors it was not possible to investigate the precise nature of the dependance of Δb on b using the present data.

The Nature of the Current Yielded by Antibody-Hapten Complexes

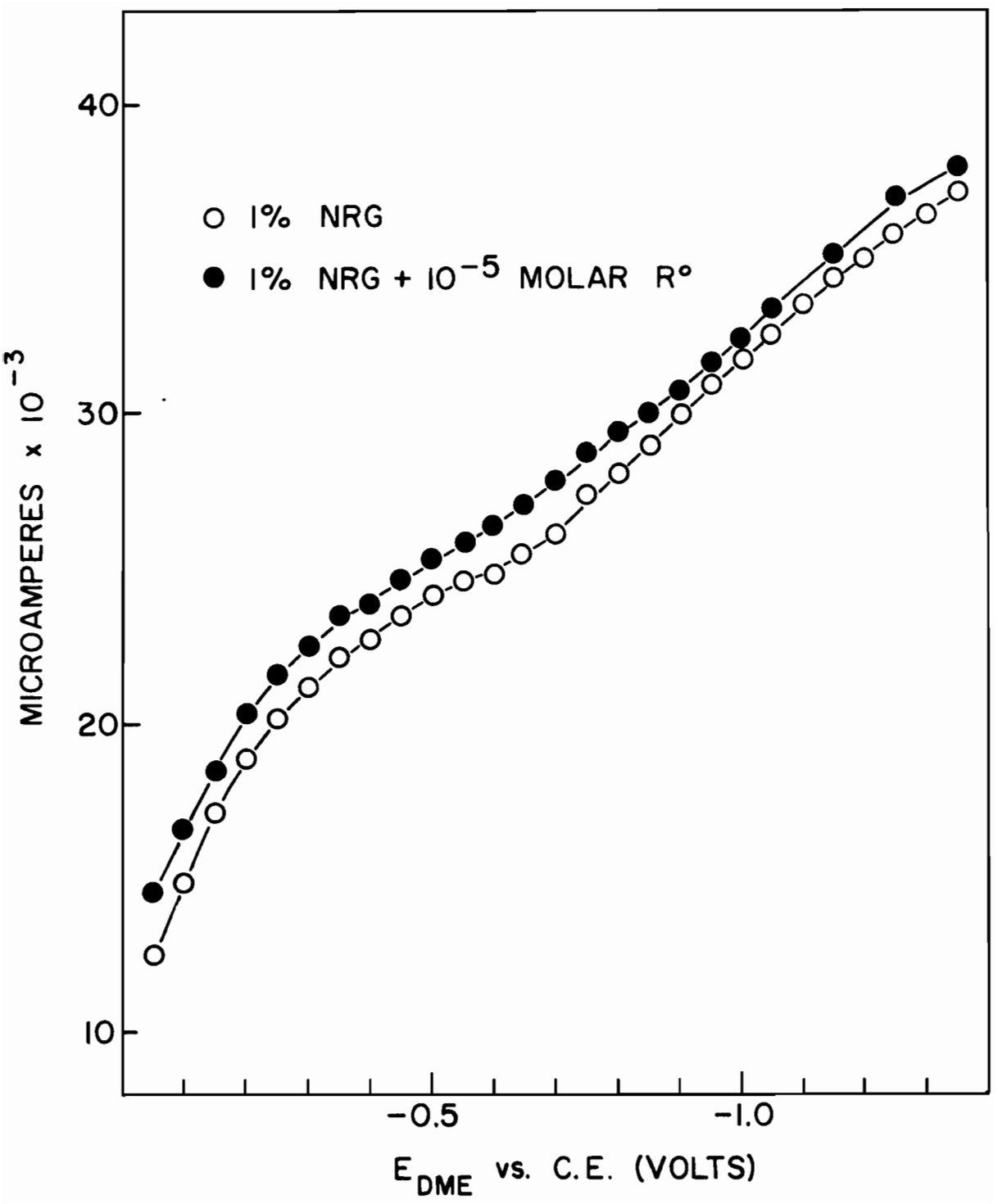
As stated above, antibody-hapten complexes may yield a current at the dropping mercury electrode because they are inherently reducible. Moreover, these complexes may dissociate at the electrode surface to yield kinetic currents,

or they may dissociate at a rate faster than that which results in kinetic currents. The absence of kinetic currents was demonstrated by the fact that the oscillographic polarograms given by solutions containing a high antibody-hapten ratio, where most of the hapten is in the form of complexes, always had the normal peaked form, in contrast to the plateau form which would be expected otherwise (121). Thus, it must be concluded that the current produced by the hapten-antibody complex arises from its inherent reducibility, or from its very rapid dissociation at the surface of the electrode.

When polarographic measurements are made by conventional techniques, the magnitude of a kinetic current varies in a distinctive manner with the height of the mercury reservoir (108). Unfortunately, the haptens did not yield well defined diffusion currents in the protein solutions in the concentration range of interest (10^{-6} to 2×10^{-5}M) as indicated in Fig. 33 by a typical polarogram obtained with R^{o} in the presence of normal gamma-globulin. Therefore, additional information concerning the absence of kinetic currents could not be obtained using conventional polarographic techniques.

FIGURE 33

CONVENTIONAL POLAROGRAMS OBTAINED WITH R^{o}
IN THE PRESENCE OF NORMAL RABBIT GAMMA-GLOBULIN


40
30
20
10
MICROAMPERES x 10^{-3}
○ 1% NRG
● 1% NRG + 10^{-5} MOLAR R°
−0.5
−1.0
E_{DME} vs. C.E. (VOLTS)

DISCUSSION

The primary reason for investigating the applicability of oscillographic polarography to the measurement of the extent of hapten binding by antibodies was the possibility that it could be used for measuring the speed of these reactions. As has been shown, it was established that one could calculate the extent of the combination of the reducible hapten with homologous antibodies by using suitable experimental procedures and precautions necessary to eliminate complications arising from the adsorption of protein on the dropping mercury electrode. On the assumption that the peak current was caused only by reduction of the unbound hapten, values for the free and bound hapten were computed which were within about 10 and 17% of the values measured by equilibrium dialysis in the B-50(5) and B-50(6) systems, respectively. These errors could be further minimized using appropriate empirical correction factors derived from equilibrium dialysis experiments. Thus, it is evident that using the procedure developed in this study, cathode ray polarography can be used to measure the reaction between reducible haptens and homologous antibodies. However, this procedure cannot be used as an independent method for the calculation of the true binding constants and for the interpretation of the results one must lean heavily on equilibrium dialysis. In other words, for the successful use of

polarography it is necessary to measure the extent of binding also by another method, and therefore, this limitation may outweigh its obvious advantages which are (i) the speed of measurement and (ii) the need for only a small amount of antibody. Nevertheless, it can be stated at this point that the necessity of interpreting the results for the extent of binding by reference to another method, such as equilibrium dialysis, does not limit the usefulness of oscillographic polarography for kinetic studies. The factor of prime importance in kinetic studies is the speed with which the experimental parameter related to the concentration of the species of interest can be determined and not the time it takes to obtain auxiliary data required for the computation of the final results.

In order to explain the origin of the discrepancy between the results for the extent of binding given by polargraphy and equilibrium dialysis, it was suggested that the bound form of the hapten yielded a faradaic current, while the observed peak height was attributed only to reduction of the unbound form of the hapten. Support for this view can be found in other studies demonstrating that complexes consisting of proteins and low molecular weight depolarizers may yield a faradaic current at the DME (142, 157). Since kinetic currents were shown to be absent in the polarograms given by mixtures of antibody and hapten, it was concluded that the current yielded by the antibody-hapten complex must

be due to its inherent reducibility or to its dissociation at the electrode surface at a very rapid rate.

In other systems where protein-depolariser complexes yielded a current it was possible to compute the concentration of the various species using an empirical correction term analaguous to that employed in the present study (143), or by solution of the two simultaneous equations which apply to such systems (142)

$$i_p = Mc + Nb \qquad (1)$$

$$c_t = c + b \qquad (2)$$

In these equations, i_p represents the faradaic current (i.e., the peak or diffusion current), while M and N represent the current yielded at a given DME per unit concentration of the free and bound depolarizer, respectively. The terms c and b represent the concentration of the free and bound depolarizer respectively. If the above equations could be used to evaluate b and c from measurements of i_p only, the disadvantages of the polarographic procedure as a general analytical method for measuring hapten binding would be eliminated to a large extent. The use of the above equations requires that the values of M and N be known. Therefore, the applicability of this method to antibody systems centers about the question as to whether or not these parameters can be determined on the basis of peak height measurements.

The constants M and N represent the peak heights yielded per unit concentration of free and bound forms of the hapten,

respectively. Therefore, each of these constants is equal to the slope of a plot of the peak height versus the concentration of the free and of the bound hapten, respectively. The corrected calibration curves represent plots of the peak height in the antibody solution yielded by the unbound hapten and, possibly, by the hapten which is bound non-specifically. Therefore, it is feasible to evaluate M from these curves. However, in practise, it would still be necessary to evaluate N, and there does not seem to be a simple method for doing this using without recourse to an independant procedure for the measurement of binding constants. Moreover, an additional complication would arise in a heterogeneous antibody system since N might not be a true constant. Consequently, the above equations cannot be used without reservation.

CHAPTER 5

A STUDY OF THE RATE OF ANTIBODY-HAPTEN REACTIONS USING OSCILLOGRAPHIC POLAROGRAPHY

Introduction

As indicated in Chapter 1, a good deal of information concerning the forces involved in hapten-antibody reactions has been obtained from thermodynamic studies. However, in general, it is not possible to derive all the information necessary to formulate the detailed mechanism of a reaction from equilibrium studies, such information being usually obtained from kinetic data. Prior to the present investigation the specific speed of antibody-hapten reactions had not been measured, although several studies of the rates of antibody-antigen reactions had been carried out (66-70). These studies indicated that the combination of haptens with antibodies would be rapid. Accordingly, an attempt was made in the present investigation to study the kinetics of such reactions using a polarographic method which allowed one to determine the concentration of reducible haptens at intervals of time as small as one second. The use of this method depended upon being able to correlate the changes in faradaic current resulting from the formation of hapten-antibody complexes with their concentration, and, as was shown in the previous section, it was found in this study that this could be done if appropriate experimental techniques were employed.

Experimental

Antibodies and Haptens

The antibodies and haptens employed were the same as those used previously to investigate the applicability of the polarographic method for measuring the extent of hapten binding. The characteristics of these antibody-hapten reactions, as determined by equilibrium dialysis, are summarized in Table II.

Methods

The apparatus used has been described in chapter 4 . In experiments designed to measure the rate of reaction of hapten with antibody, hapten solutions were added to the antibody solution which was being stirred continuously. Measurements of peak height were then made as soon as possible thereafter at intervals of one second. Both the hapten and antibody solution were deoxygenated before mixing using the techniques described previously. The sweep rate employed was 10 volts/second.

The peak height which was obtained in successive readings when measurements were made under the conditions employed for equilibrium measurement was reproducible to $\pm 2\%$. In the stirred solutions employed for rate experiments, the peak height was reproducible to $\pm 8\%$.

Results

(a) The Forward Reaction

In experiments designed to measure the rate of the for-

ward step in the reactions

$$Ab + R^{o} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} Ab - R^{o} \qquad (1)$$

$$Ab + Res\text{-}R \underset{k_r}{\overset{k_f}{\rightleftharpoons}} Ab - Res\text{-}R \qquad (2)$$

an aliquot of the reducible hapten was rapidly added to the antibody solution and the peak height was observed. The final concentration of both antibody and hapten were about 10^{-5}M in these experiments. After the time of mixing, estimated to be 1 to 2 seconds, there was no detectable trend in the peak height with time. Thus, within experimental error, the association reaction between antibody and hapten had reached completion within this interval and, therefore, the rate constant k_f could not be evaluated directly. However, since the intrinsic equilibrium constant for the antibody-hapten reaction, determined previously as $\sim 10^{6}M^{-1}$, is equal to the ratio of the two rate constants in reaction (1) and (2), that is to k_f/k_r, it is possible to evaluate k_f by first determining k_r. Accordingly, attempts were made to determine k_r.

(b) The Reverse Reaction

In an attempt to determine k_r advantage was taken of the facts that the reducible hapten can be released from the antibody site by suitable amounts of benzenearsonic acid (BAA), and that the amount of the former can then be

measured polarographically. This reaction is illustrated in Fig. 34 which shows how the peak height steadily increases on the addition of a concentrated solution of BAA to the equilibrated solution of the antibody and the reducible hapten. As mentioned previously, the compound BAA was structurally closely related to the reducible hapten, and, thus, could compete with the latter for the antibody site. It is of interest to note, as can be seen also from Fig. 34, that the peak height increased steadily as BAA was added, and that eventually it reached asymptotically the value identical to that given by the calibration curve. This fact provides additional evidence for the reversibility of antibody hapten reactions.

The fact that BAA can replace the reducible hapten was exploited for the derivation of kinetic data on the basis of the following two assumptions:

(1) the overall substitution reaction

$$Ab - R^{o} + BAA \longrightarrow Ab - BAA + R^{o}$$

is a two step process consisting of the slow, first-order dissociation reaction

$$Ab - R^{o} \xrightarrow{k_r} Ab + R^{o} \qquad (1^1)$$

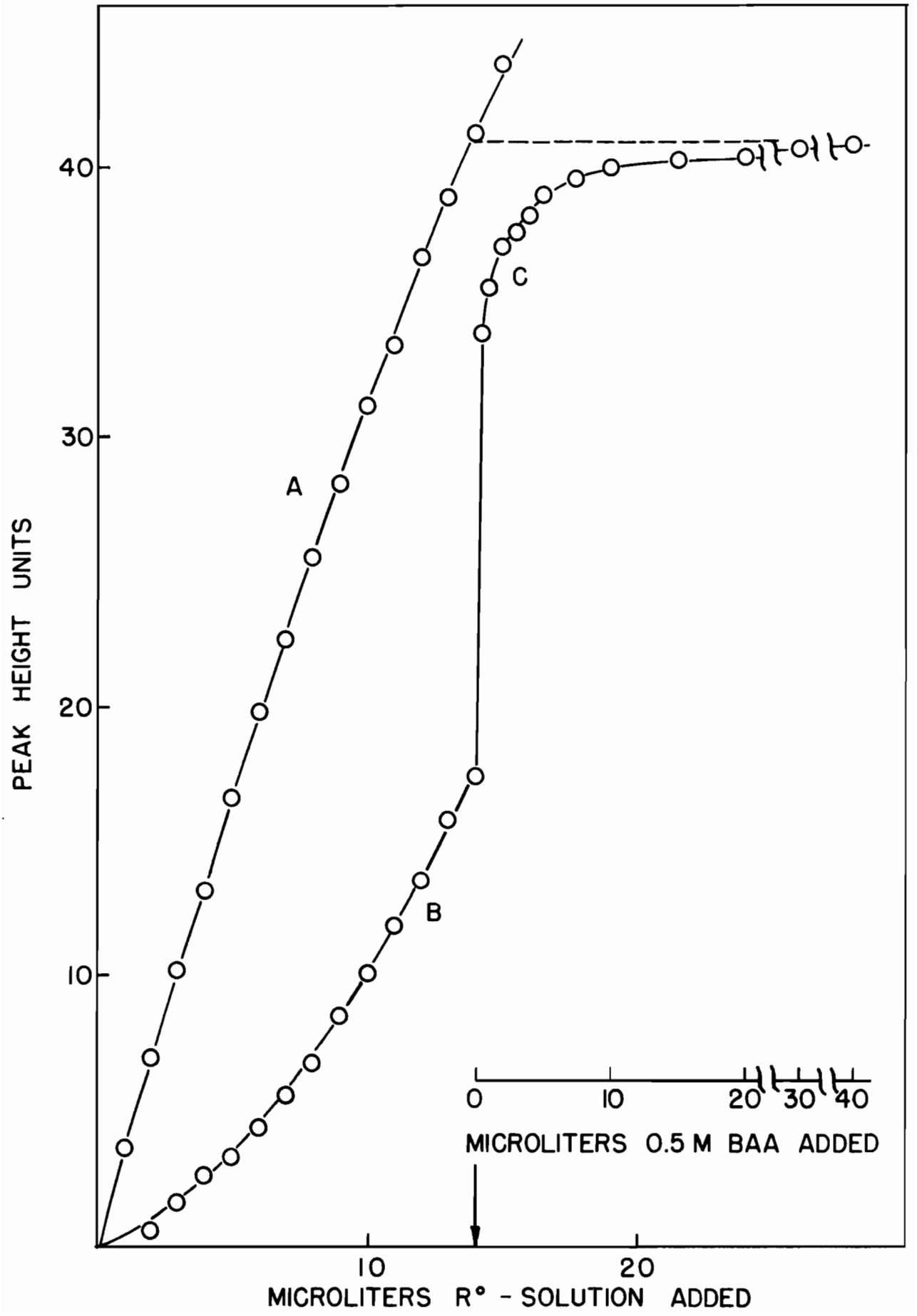
which is followed by the rapid reaction

$$Ab + BAA \xrightarrow{k_f^1} Ab - BAA \qquad (2^1)$$

FIGURE 34

INHIBITION OF THE REACTION BETWEEN R^{o} AND THE HOMOLOGOUS ANTIBODIES BY BENZENEARSONIC ACID

Line A represents the calibration curve; line B, the antibody titration curve; line C represents the peak height measured when various amounts of benzenearsonic acid (BAA) were added to an equilibrated solution of antibody and hapten. The volume of antibody solution used in obtaining these curves was 1.04 ml. The concentration of the R^{o} solution added was 1.04×10^{-3} molar.


PEAK HEIGHT UNITS
40
30
20
10
A
B
C
0
10
20
30
40
MICROLITERS 0.5 M BAA ADDED
10
20
MICROLITERS R° - SOLUTION ADDED

(2) The rate constants for the reaction between the antibody molecules and R^o on the one hand, and BAA on the other, are of the same order of magnitude (that is, $k_f \simeq k_f^1$).

If these assumptions are correct, then, on the addition of sufficiently large amounts of BAA to an equilibrated solution of antibody and R^o, the increase in peak height with time would represent the rate of the dissociation step (1^1). In accordance with the above scheme, experiments to determine the value of k_r were performed by adding microliter volumes of a 0.5M solution of BAA to solutions of antibody and R^o or Res-R. The final ratios of BAA to reducible hapten used varied from 25 to 1000 and the total concentration of the latter was 10^{-5} or 5×10^{-6} molar. In each case the peak height due to R^o increased within the time necessary to make the first observation after the addition of BAA, and, within experimental error, further changes in its value could not be detected for periods of time as long as half an hour. Therefore, it was concluded that the dissociation reaction, like the association reaction, was completed within 1 to 2 seconds.

Although the dissociation reaction was too rapid for the precise determination of its rate, a <u>lower limit</u> for k_r could readily be calculated with the aid of the standard expression for a first-order reaction $k = (\frac{1}{t}) \ln (\frac{a}{a-x})$.

If t is taken as 1 sec., it follows that k_r must be larger than 1 $sec.^{-1}$. Using this estimate together with the value for the intrinsic equilibrium constant, the minimum value for k_f was calculated as 10^6 l/mole.sec.

DISCUSSION

Although the speed of antibody-hapten reactions could not be measured directly using the polarographic method, the lower limit for the rate constant for the forward reaction was found to be 10^6 l/mole/sec.

For most bimolecular, diffusion-controlled reactions between relatively small molecules in solution, rate constants of the order of 10^9 - 10^{11} $M^{-1}sec^{-1}$ can be calculated (159). Alberty and Hammes (158) adopted the existing theoretical expressions for diffusion-controlled, bimolecular reactions to enzyme-substrate interactions 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^2sec^{-1} and 4×10^{-7} cm^2sec^{-1} for the hapten and antibody molecules, respectively. Because of steric factors and electrostatic repulsion arising from the fact that at pH8 both reactants are negatively charged,

it is likely that the actual value of k_f is smaller than this maximum value by a factor of at least 10. Therefore, on the basis of the experimental results and of the above calculation, one can reasonably predict the true value of k_f to be between the limits of 10^6 and 10^8 l/mole/sec.

In principle, the speed of a reaction with a rate constant greater than 10^6 l/mole/sec. can be measured using (by now) conventional rapid mixing methods, when sufficiently low concentrations of reactants can be employed, or by the use of relaxation techniques developed by Eigen et al. (107). With the temperature-jump relaxation method the rate of reactions occuring within one microsecond to onesecond can be measured, provided the reactions are associated with a change in enthalpy. In brief, this method involves raising the temperature of the reaction system within 0.1-1 μsec by discharging a condensor through a conducting solution containing the system under study. The re-attainment of equilibrium at the higher temperature is then followed by some convenient physical method, such as optical density or conductivity measurements.

Subsequent to the publication of the results obtained in the present study (105, 106), the speed of hapten-antibody reactions were actually measured by both methods mentioned. Froese, Sehon and Eigen (103), used the temperature-jump method and determined k_f and k_r for the reaction between rabbit antibodies specific to the azophenylarsonate ion and

a hapten containing this group to be 2 x $10^7 M^{-1} sec.^{-1}$ and 50 sec^{-1} respectively. This reaction was followed spectrophotometrically by exploiting the fact that the absorption spectrum of the hapten used, 1-napthol-4-[4-(4^1-azobenzeneazo)-phenylarsonic acid], changed when it combined with the antibody site.

Day, Sturtevant and Singer (78,79) and Sturtevant, Wofsy and Singer (104) used a stopped-flow method and determined k_f to be about $10^8 M^{-1} sec.^{-1}$ for the combination of antibodies to the dinitrophenyl group (DNP) with DNP-lysine, DNP-aminocaproate and 2-(2,4-dinitrophenylazo)-1-napthol-3,6 disulfonic acid. The value of the rate constant for the reverse reaction k_r, was estimated to be 1 $sec.^{-1}$ from the relation $K_{eqbm} = \frac{k_f}{k_r}$. In these studies, the reaction of the first two of the haptens listed above was followed by measuring the changes in antibody fluorescence which occured when the hapten and antibody combined. This method is very sensitive, allowing concentrations of the order of $10^{-7} M$ to be used; thus, the stopped flow method could be used to obtain precise kinetic data. The reaction involving the last of the haptens listed was followed by a spectrophotometric method similar to that used by Froese et al. (103). Since this method is not as sensitive as that employing fluorescence measurements, sufficiently low concentrations could not be employed, and only the combination of reactants in the latter stages of the reaction could be measured.

The energies of activation for the reactions of DNP-lysine and DNP-aminocaproate with the homologous antibodies were calculated by Day et al. (79) from the temperature coefficient of k_f as 4.1 ± 1.0 kcal/mole and 4.4 ± 1.0 kcal/mole, respectively. These values are comparable to the value of 4-5 kcal for the energy of activation of viscous flow in aqueous media (111), and it was suggested that these reactions were diffusion controlled (79). The small energy of activation has also been considered to mean that substantial conformational changes do not occur when the hapten is bound (79).

It is of interest to note that the values of k_f determined by Froese et al. (103) and Day et al. (79) for two different antibody-hapten systems were similar, although the intrinsic equilibrium constants for association for these systems differed by almost three orders of magnitude ($5 \times 10^5 M^{-1}$ and $\sim 10^8 M^{-1}$, respectively). This result suggests strongly that the rate of the dissociation reaction might be much more sensitive to the particular environment created by the antibody site for the hapten, since the forward reaction appears to be diffusion controlled. Thus, it is conceivable that more information concerning the mechanism of antibody-hapten reactions at a molecular level could be obtained by studying the dissociation reactions rather than the association process.

Since the population of antibodies in an antiserum is usually heterogeneous with respect to their binding affinity

for haptens (c.f. Chapter 1, page17) Froese, Sehon and Eigen (103) suggested that this should be reflected also in a corresponding statistical distribution of k_f and k_r values. However, only single values for k_f and k_r were determined by these authors in the system studied although reactions with rate constants as large as 10^9 l/mole/sec could have been detected by the temperature-jump technique used (107). On the other hand, Day, Sturtevant and Singer (79) detected a gradation in their rate constants. Thus, when the reactions were studied using a high ratio of hapten concentration to that of antibodies, it was found that k_f decreased as the time of reaction increased. This variation in the values of k_f may be due to at least three possibilities: (i) the two binding sites may not act independently from each other; (ii) there may be a single value for the rate constant k_f and a distribution of values for k_r; (iii) there may be a distribution for both k_f and k_r. Since Nisonoff et al (156) have shown that the average intrinsic equilibrium constant and index of heterogeity of univalent antibody fragments is the same as that of the intact divalent antibody, it is likely that the first of the above possibilities is not responsible for the observed heterogeneity in the rate constant. At the present time the data necessary to distinguish between the other two possibilities are not available.

GENERAL DISCUSSION

Although it was shown that the polarographic method developed for following the combination of reducible haptens with antibodies gave results for the concentration of bound hapten which were lower than those given by equilibrium dialysis by 10 to 17%, it was also shown that the polarographic data could easily be corrected to yield more satisfactory results using an empirical correction factor derived from parallel experiments with equilibrium dialysis and polarography. Thus, although the polarographic method is not suitable as a general and independent method for establishing the extent of antibody-hapten reactions, it may yield sufficiently satisfactory results in instances where other methods cannot be used. This might occur, for example, when the hapten of interest will not pass through or is bound irreversibly to the membrane used in equilibrium dialysis experiments, or when the hapten does not have an absorption spectrum appropriate for its use with the fluorescence quenching technique.

In general, one of the difficulties associated with the use of polarography for the study of interaction of low-molecular weight depolarizers with proteins are the complications caused by the adsorption of the latter on the mercury electrode (138). In the present study these difficulties were overcome by obtaining an appropriate calibration

curve. This was possible since the ability of the antibody to bind the hapten could be virtually eliminated by the addition of an appropriate inhibitor, without affecting in a pronounced manner the effects caused by the adsorption of protein on the faradaic currents of depolarisers. It is obvious that this method is not restricted to antibody-hapten systems and may prove useful in other polarographic studies of reactions involving proteins.

In the oscillopolarograms given by the gamma-globulin solutions a small peak was observed at a potential of about -0.5 volts versus the saturated calomel electrode which may have been due to the reduction of some of the intramolecular disulfide bonds of these molecules. If this is the case, then there is the implication that at least some of the disulfide bonds are at the surface of the molecule, and this idea is supported by the finding that some of these groups are split by simple thiols much more readily than others (47, 117). Accordingly, it might be feasible to establish with polarography if a protein has disulfide groups near the surface, a fact which would be of obvious interest in studies of its structure.

In order to explain the low values for the bound hapten concentration obtained by polarography it was suggested that the hapten may be reduced while it is complexed with the antibody, or that it cannot be reduced in this form but may dissociate rapidly at the electrode and then be reduced.

In principle, it is feasible to measure the rate of such dissociation reactions using polarographic methods, provided the rate is of the order of that required to yield a kinetic current (108). However, no evidence was found for the presence of kinetic currents in the present study.

Although the absolute speed of antibody-hapten reactions could not be measured directly with the polarographic method employed, it was shown that k_f lies in the rangeof 10^6 to 10^8 1/mole/sec. Moreover, this result indicated that these reactions could be studied by more rapid techniques, such as rapid flow methods or the temperature-jump relaxation method. This conclusion was subsequently confirmed by other workers (78, 79, 103, 104), who evaluated k_f to the $2 \times 10^7 - 10^8$ 1/mole/sec. These values for k_f, together with the limiting values estimated in the present investigation, indicate that all antibody-hapten reactions are extremely fast, these reactions being among the fastest bimolecular, homogenous reactions in solution involving protein molecules.

Because the values of k_f and of the activation energy were found to be close to those which would be expected for a diffusion controlled reaction, Day et al. (79) suggested that the rate determining step is diffusion of the hapten to the antibody site. However, the role played by the steric and electrostatic factors has yet to be established. Undoubtedly, future work will be directed towards the elucidation of these

factors and should involve studies of the effects of pH, ionic strength, dielectric constant of the medium and its viscosity on the rates and equilibria of these reactions. Furthermore, it would also be of interest to compare the rate constants for the reaction between univalent antibody fragments and haptens with those for the intact, bivalent antibody molecule.

SUMMARY

(1) An oscillographic polarograph was built for measuring the change in concentration of reducible haptens in the micromolar range within intervals of time as short as one second.

(2) Two readily reducible azo derivatives of benzearsonic acid, p-(p-aminophenylazo)phenylarsonic and p-(2,4-dihydroxyphenylazo) phenylarsonic acid (referred to as R^o and Res-R, respectively), were shown to combine specifically with rabbit antibodies to the p-azophenylazophenylarsonate group.

(3) The oscillographic polarograms given by the two azo compounds Res-R and R^o in a borate buffer, pH8.0,μ=0.15 in the absence of proteins and in the presence of normal rabbit gamma-globulins as well as the specific antibodies were determined.

(4) It was shown that normal rabbit gamma-globulins were adsorbed on the mercury drops at pH 8.0, and this adsorption resulted in a measurable decrease of the faradaic current yielded by R^o and Res-R in the presence of normal gamma-globulins.

(5) The combination of the reducible compounds R^o and Res-R with the homologous anti-hapten antibodies resulted in a further, marked decrease in faradaic current.

(6) A calibration technique was developed to account for and eliminate the complications due to the adsorption of gamma-globulins on the dropping mercury electrode.

(7) The concentration of bound hapten determined polarographically was 10 - 17% lower than that yielded by the method of equilibrium dialysis. It was suggested these lower results were due to the reduction of some of the haptens in the corresponding complexes with antibodies.

(8) The polarographic data could be adjusted to yield more accurate results using an empirical correction factor derived in parallel equilibrium dialysis and polarographic experiments.

(9) The intrinsic equilibrium constant determined by polarography and by equilibrium dialysis for the reaction of R^o with the homologous antibodies was found to be 6.65×10^5 and 8.30×10^5 1/mole, respectively. The corresponding values for the experiments with Res-R were 6.40×10^5 and 28.0×10^5 1/mole, respectively.

(10) The index of heterogeneity determined from the equilibrium dialysis data using the method of Sips was 0.7 for the reaction of Res-R with the appropriate antibodies. There was no appreciable heterogeneity in the reaction of R^o with antibodies.

(11) Using the polarographic procedure developed, the forward as well as the reverse step in the reactions $Ab + H \underset{k_r}{\overset{k_f}{\rightleftharpoons}} AbH$

was shown to be completed within less than 1-2 seconds. Lower limits of 10^6 l/mole/sec and 1 sec^{-1} were calculated for the rate constants of the two reaction steps, i.e. for k_f and k_r.

(12) It was deduced that the actual value of k_f should be within the range 10^6 -10^8 l/mole/sec. This conclusion was recently confirmed by two other groups of workers using rapid flow and relaxation techniques.

CLAIMS TO ORIGINALITY

1. Normal rabbit gamma-globulins were shown to be adsorbed on a mercury surface at pH 8.0.

2. The faradaic current yielded at a dropping mercury cathode by the two azo compounds p-(p-aminophenylazo)phenylarsonic acid (R^o) and p-(2,4-dihydroxyphenzlazo)phenylarsonic acid (Res-R) was shown to be decreased by the adsorption of normal rabbit globulins on the mercury drops.

3. The faradaic currents yielded by R^o and Res-R were shown to decrease upon combination with anti-hapten antibodies specific for the p-azophenylazophenylarsonate group.

4. It was shown that by using a suitable calibration method, the change in faradaic current due to the formation of antibody-hapten complexes could be differentiated from the change due to adsorption, and therefore, that it was feasible to use polarography for measuring the extent of hapten binding by antibodies. The concentration of bound hapten measured by polarography was found to be 10 - 17% lower than the corresponding value measured by equilibrium dialysis. However, more accurate results could be obtained by the application of a correction factor determined in parallel experiments with polarography and equilibrium dialysis.

(5) Using the polarographic procedure, the forward as well as the reverse step in the reactions $Ab + H \underset{k_r}{\overset{k_f}{\rightleftharpoons}} AbH$ was shown to be completed within less than 1-2 seconds. Lower

limits of 10^6 l/mole/sec. and 1 sec^{-1} were calculated for the rate constants of the two reaction steps, i.e. for k_f and k_r. It was deduced that the actual value of k_f should be within the range 10^6 - 10^{-8} l/mole/sec.

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