Thermodynamics of Antibody–Antigen Reactions. 2. The Binding of Bivalent Synthetic Random Coil Antigens to Antibodies Having Different Antigen Precipitating Properties†

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ABSTRACT: The objects of this study were the equine IgG and IgG(T) classes of antibodies with immunologic specificity for the dinitrophenyl group and bivalent antigens consisting of linear poly(ethylene glycol) polymers which terminated at both ends in dinitrophenyl groups. Complex formation between antibodies of both classes and one of several sharp fractions of antigen having number average molecular weights in the range 25 000 to 75 000 were studied by measuring the light scattered from solutions containing equimolar amounts (~5 $\times 10^{-6}$ mol/L) of one of the antibodies and one size fraction of antigen, and variable amounts of monovalent hapten. The data were analyzed in the context of a model that accounted for the formation of linear and cyclic complexes of all extents of aggregation. Two parameters in addition to the intrinsic antibody-dinitrophenyl group association constant were found to be necessary in the assumed equilibrium model to account for the behavior of the system. One of these accounted for the losses in configurational entropy that resulted when a random-coil polymer became bound at one end to a space-occupying antibody. The other was a ring closure factor for the formation of cyclic complexes. Ring closure factors for the formation of larger cyclic complexes (present in only small amounts under the conditions studied) were related to the ring closure factor for the formation of the smallest, which was found to increase as antigen size decreased, and for each antigen size to be consistently higher for IgG(T) antibody than for IgG antibody. Comparisons of the theoretically estimated values of the two parameters with their measured values indicated that the average conformation of IgG antibodies in solution is open ("T" shaped), but the average inter-Fab arm angle in IgG(T) antibodies is approximately 60° or less.

As the biological roles of antibodies have become better understood, it has become apparent that some immunological phenomena such as the clearance of antigen–antibody complexes from the circulation (Mannik et al., 1971), their deposition in organs and tissues in certain diseases (Cochrane and Hawkins, 1968), and the initiation of the sequence of reactions involving the components of the complement system (Augener et al., 1971) are very dependent on the size and composition of the complexes, and that the nature of these complexes in turn depends very much on the class of antibody incorporated in them (Klinman and Karush, 1967). The varying ability of different classes of antibodies which are alike in their specificity, valency, and intrinsic site binding affinity to precipitate with multivalent antigens suggests that some classes may bind more readily with both sites to the same multivalent antigen, and other classes may instead preferentially link two antigen molecules together. This suggestion has been provided a theoretical basis by a development of DeLisi (1974) which predicts that the tendency of an antibody to bind bivalently to an antigen will restrict the conditions under which precipitation is possible and therefore influence the size and nature of the complexes also. There is a growing number of species in which a class of bivalent antibodies is known that precipitates with antigens only within a very narrow range of antibody and antigen concentrations, or not at all. The first and best known of these so-called "nonprecipitating" antibodies is the equine IgG(T) class. Klinman and Karush (1967), in a study of the binding behavior of equine antibodies to antigens attached to a solid phase, have shown that IgG(T) antibodies have a greater tendency than the precipitating IgG classes to bind with both sites to a multivalent antigen. This information led them to suggest that the Fab arms, which bear the specific binding sites, might be positioned closer together in IgG(T) than in IgG. This hypothesis is consistent with the fact that an additional interheavy chain disulfide bridge is present in the hinge of IgG(T) antibodies (Raynaud and Iscaki, 1964). However, partial reduction and alkylation of the interchain disulfides in IgG(T) does not affect their behavior (Klinman and Karush, 1967).

The subject of this report is a study of these two classes of equine antibodies in which we have obtained values for the equilibrium constant for the reaction

$$\text{habag} \rightleftarrows \text{ab}{=}\text{ag} + \text{h} \qquad (1)$$

in which a bivalent antibody, ab, bound at one site to a bivalent antigen, ag, and at the other to a monovalent hapten, h, releases the hapten and forms a ring with the antigen. These equilibrium constants depend on both the average intersite distances in the antibodies and the configuration and size of the antigens. Differences in the equilibrium constants for the formation of ring complexes between IgG or IgG(T) and antigens of various sizes point to substantial differences in the average distances between the binding sites of IgG(T) antibodies and therefore to significant differences in the conformation of these antibodies in solution.

Experimental Procedures

Antibody. The antibodies were produced in Shetland ponies

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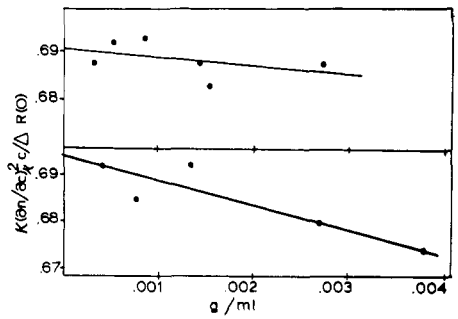


FIGURE 1: Light-scattering measurements on IgG(T) antibody (top) and IgG antibody (bottom) in PKBS. The indicated lines are the results of least-squares analyses.

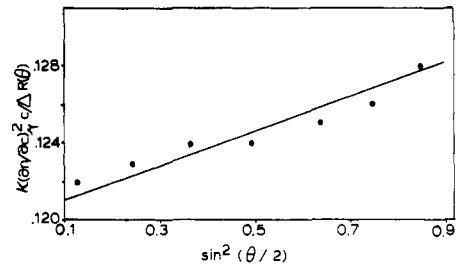


FIGURE 2: The angular dependence of scattered light for an antigen fraction with a number average molecular weight of 74 800 at a concentration of 0.00151 g/ml. The indicated line is the result of a least-squares analysis.

and were purified by procedures described elsewhere (Archer et al., 1973). Their concentrations were determined using molar absorbancies at 278 nm, for IgG of $(1.941 \pm 0.010) \times 10^5$ L mol^{-1} cm^{-1}, and for IgG(T), $(1.880 \pm 0.009) \times 10^5$ L mol^{-1} cm^{-1}. These values were determined from measurements of the light scattered by solutions of purified antibodies at various concentrations, according to the equation

$$1000\kappa\left(\frac{\partial n}{\partial A_\lambda}\right)_\mu^2 \frac{A_\lambda}{\Delta R(0)} = \frac{1}{\epsilon_\lambda} + 2B^c A_\lambda + \ldots \qquad (2)$$

where $\Delta R(0)$ is the reduced intensity of light observed at an angle of zero degrees to the incident beam, A_λ is the measured absorbance of the solution, κ is the optical constant of the photometer, ϵ_λ is the molar absorbancy of the antibody, and B^c is the second virial coefficient of the antibody. Absorbancies on a weight-per-volume concentration scale were determined by drying at 80 °C to constant weight solutions of antibodies that had been freed of all counter- and coions except H^+ and OH^- by passage over two mixed-bed ion-exchange columns (Edsall and Wyman, 1958). The values obtained were: for IgG, 1.352 ± 0.015 L g^{-1} cm^{-1}, and for IgG(T), 1.306 ± 0.015 L g^{-1} cm^{-1}. Molecular weights for the two antibodies, excluding all salt counterions calculated from the absorbancies on the two concentration bases, are 143 600 ± 1800 g/mol for IgG and 144 000 ± 1800 g/mol for IgG(T). Properties of the antigen to be described below dictated that all light-scattering experiments be done on solutions containing 0.45 M K_2SO_4. The solutions also were buffered at pH 7.8 by 0.01 M sodium phosphate. This solution is referred to below as PBKS.[1] Plots of the light-scattering measurements are shown in Figure 1. Each point has an uncertainty of 1.0–1.5%.

Haptens. The monovalent hapten Dnp-MP was prepared as has been described (Cooke et al., 1974).

Antigens. Bivalent antigens consisting of linear polymers of poly(ethylene glycol) terminating at both ends by Dnp groups were made by procedures already described (Cooke et al., 1974) from a sample of high-molecular-weight Carbowax poly(ethylene glycol), 68 000, which was a gift from the Union Carbide Co. Fractionated samples of PEG-Dnp_2 were characterized by their number- and weight-average molecular weights, $\overline{M}_n$ and $\overline{M}_w$, respectively. The molecular weights of the antigen fractions used are listed in Table I. $\overline{M}_n$ was determined by the ratio of concentrations in terms of mass and moles per liter, the former determined by differential refractometry, and the latter, spectrophotometrically, using the extinction coefficient of Dnp-MP. $\overline{M}_w$ and the mean square radius of gyration, R_G^2, were measured by light-scattering photometry making use of the expression (Tanford, 1961, p 161),

$$\lim_{c\to 0} \frac{\kappa(\partial n/\partial c)_\mu^2 c}{\Delta R(\theta)} = \frac{1}{\overline{M}_w}\left(1 + \frac{16\pi^2}{3\lambda'^2}\langle R_G^2\rangle \sin^2\frac{\theta}{2} + \ldots\right) \qquad (3)$$

where λ' is the wavelength of the incident light within the scattering medium, θ is the angle of observation with reference to the incident beam, and c is the concentration in grams per milliliter. R_G^2 is measurable only for those antigens which are large enough that sufficient intramolecular interference occurs to result in a measurable dependence of $\Delta R(\theta)$ on θ. Careful corrections for distortions in the scattering envelope of the cell and photometer were applied. In polymer samples that consist of a distribution of molecular weights, the z average of R_G^2 is obtained (Tanford, 1961, p 167); however, the final fractions used in these experiments were sharp enough that differences in averaging could be ignored (see Table I). The data from measurements on a large antigen are shown in Figure 2. PBKS was the solvent in all measurements because at 25 °C it is nearly an ideal solvent for PEG; i.e., PEG behaves nearly thermodynamically ideally in it and assumes a compact form (Beech and Booth, 1969). All antigen preparations were stored in the dark at 4 °C in an atmosphere of nitrogen with 1% 2-propanol added as an antioxidant.

Refractometry. Refractive increments were measured in a Brice-Phoenix differential refractometer. The quantities $(\partial n/\partial c_2)_{\mu_3}$ (Casassa and Eisenberg, 1964), which represent the dependence of the refractive index of a solution on the concentration (in grams per milliliter) of a macromolecular component while all diffusible solute chemical potentials are held fixed, were obtained from measurements of the difference in refractive index between a solution containing antibody or antigen and one whose diffusible components were at the same

TABLE I: Molecular Properties of the Fractionated Antigens.[a]

$\overline{M}_n$ (g/mol)	$\overline{M}_w/\overline{M}_n$	$\langle R_G^2\rangle^{1/2}$ (nm)
74 800 ± 1000	1.13 ± 0.04	12.2 ± 1.3
63 000 + 900	1.06 ± 0.03	11.3 ± 3.1
62 200 ± 1200	1.04 ± 0.03	18.0 ± 2.0
57 800 ± 800	1.00 ± 0.04	8.4 ± 6.3
45 400 ± 700	0.97 ± 0.03	8.8 ± 4.5
35 600 ± 700	1.03 ± 0.04	
25 000 ± 1200	ND	

[a] Listed uncertainties are standard errors. ND, not determined.

[1] Abbreviations used: Dnp, dinitrophenyl, MP, methoxypoly(ethylene glycol) 750, PEG, poly(ethylene glycol); PBKS, 0.45 M K_2SO_4 buffered by 0.01 M sodium phosphate (pH 7.8).

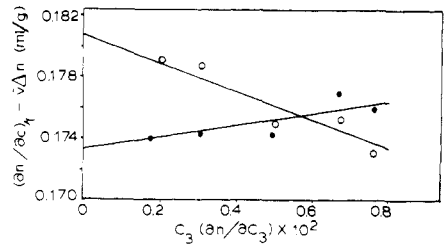


FIGURE 3: The dependence of the refractive increments at fixed chemical potential of K_2SO_4 for IgG(T) (open circles) and IgG (closed circles) antibodies on the concentration of K_2SO_4. The indicated lines are the results of least-squares analyses.

chemical potential but containing no macromolecule. Such solution pairs were obtained by passing a solution of antibody or polymer through a column of Bio-Gel P-6, maintained at 25 °C and equilibrated with PBKS. The values determined for IgG, IgG(T), and PEG polymer were 0.1736 ± 0.0019, 0.1762 ± 0.0014, and 0.1022 ± 0.0005 ml/g, respectively. The refractive increment obtained at fixed composition of the solvent, $(\partial n/\partial c_2)_{c_3}$, was easily obtained for the unmodified polymer PEG since it is a nonelectrolyte available in dry form, and the necessary solution pairs could be made up by weight. A value of the 0.1240 ± 0.0007 ml/g was measured. The molar refractive increment, $(\partial n/\partial C_2)_{C_3}$, for antigens was calculated from the approximate equation:

$$\left(\frac{\partial n}{\partial C_2}\right)_{C_{PBKS}} = \frac{\bar{M}_n}{1000}\left(\frac{\partial n}{\partial c_2}\right)_{C_{PBKS}} + 2\left(\frac{\partial n}{\partial C_{Dnp}}\right)_{C_{PBKS}} \quad (4)$$

where C_2 is the molar concentration of antigen, c_2 is the concentration of PEG in grams per milliliter, and $(\partial n/\partial C_{Dnp})$ was measured as described (Cooke et al., 1974). The contribution of the terminal Dnp groups to the molar refractive increments of the antigens was only a few percent.

The direct measurements of $(\partial n/\partial c_2)_{c_3}$ for antibody requires pairs of solutions (one containing antibody and one not) which have the same concentration of K_2SO_4. To avoid the need to obtain a concentrated solution of deionized antibody which would be required to make up these solution pairs by gravimetric or volumetric procedures, an indirect approach to the measurement of $(\partial n/\partial c_2)_{c_3}$ for the antibodies was employed. We begin with a differential expression which has been used by Noelken and Timasheff (1967)

$$\left(\frac{\partial n}{\partial c_2}\right)_{\mu_3} = \left(\frac{\partial n}{\partial c_2}\right)_{c_3} + \left(\frac{\partial n}{\partial C_3}\right)_{c_2}\left(\frac{\partial C_3}{\partial c_2}\right)_{\mu_3} \quad (5)$$

and substitute an expression for the interaction term at infinite dilution, $(\partial C_3/\partial c_2)_{\mu_3}°$, given by Casassa and Eisenberg (1964)

$$\left(\frac{\partial C_3}{\partial c_2}\right)^{\circ}_{\mu_3} = C_3\left[\left(\frac{V_m{}^{\circ}\zeta_3{}^{\circ}}{m_3}\right) - \bar{v}_2{}^{\circ}\right] \quad (6)$$

which relates the quantity $(\partial C_3/\partial c_2)_{\mu_3}$ to $\zeta_3° = (\partial m_3/\partial m_2)_{\mu_3}/M_2$. m_3 and m_2 are moles of component 3 and of component 2 per kilogram of solvent, $V_m°$ is the volume in milliliters of PBKS containing 1 kg of water, and $\bar{v}_2°$ is the partial specific volume of antibody. Another semiempirical relation obained by Casassa and Eisenberg (1964), which relates the refractive increment at fixed solvent composition in one solvent to other solvents, is also needed.

$$\left(\frac{\partial n}{\partial c_2}\right)_{c_{3a}} = \left(\frac{\partial n}{\partial c_2}\right)_{c_{3b}} - (n_a - n_b)\bar{v}_2 \quad (7)$$

n_a and n_b are the refractive indices in solvents a and b. Substituting eq 6 and 7 into eq 5, we obtain

$$\left(\frac{\partial n}{\partial c_2}\right)_{\mu_3} - \bar{v}_2\Delta n = \left(\frac{\partial n}{\partial c_2}\right)_{C_{PBKS}} + \left(\frac{\partial n}{\partial C_3}\right)_{c_2} C_3\left[\left(\frac{V_m{}^{\circ}\zeta_3{}^{\circ}}{m_3}\right) - \bar{v}_2{}^{\circ}\right] \quad (8)$$

where Δn is the refractive index of PBKS minus the refractive index of a solution with K_2SO_4 concentration, C_3. To obtain $(\partial n/\partial c_2)_{c_3}$ in PBKS, the quantity $(\partial n/\partial c_2)_{\mu_3}$ at several K_2SO_4 concentrations was measured. The value of $(\partial n/\partial C_3)_{c_2}$ was assumed equal to the value for $(\partial n/\partial C_3)$ in the absence of component 2 that can be calculated from data in Weast et al. (1970), and $\bar{v}_2$ was taken to be 0.74 ml/g (Montgomery et al., 1969). Plots of the left-hand side of eq 8 against $C_3(\partial n/\partial C_3)_{c_2}$ for both antibodies are shown in Figure 3. The apparent linear dependence of the left-hand side of eq 8 on $C_3(\partial n/\partial C_3)_{c_2}$ indicates that the value of $\zeta_3°/m_3$ is independent of m_3 for both antibodies. The interaction terms, $(\partial m_3/\partial m_2)_{\mu_3}$, which include Donnan effects, hydration effects, and all other interactions between K_2SO_4 and the antibodies are calculated from the slopes of the lines in Figure 3 to be −30 and +74 mol of salt per mol of antibody for IgG and IgG(T) antibodies in PBKS, respectively. The intercepts on the ordinate give values of 0.1808 ± 0.0011 and 0.1733 ± 0.0008 ml/g for the quantities $(\partial n/\partial c_2)_{C_{PBKS}}$ for IgG and IgG(T) antibodies, respectively.

Light Scattering. All light-scattering measurements were performed in a Brice-Phoenix photometer with 546-nm light, and a 25-mm cylindrical cell with flattened sections for the entrance and exit of the incident beam. The standard 4-mm collimating slits were replaced with 2-mm slits. Temperature control was accomplished by circulating water at 25 °C through the cell table and a jacket which surrounded the cell except for a slit to allow for the passage of incident, transmitted, and scattered light. Calibration measurements with hen-egg white lysozyme, purified by chromatography on Sephadex G-50, were done in 0.1 M NaCl buffered at pH 2.5 with 0.05 M glycine using a molecular weight for lysozyme $C1_{16}$ of 14 890, absorbancy at 280 nm of 2.613 ± 0.003 L g^{-1} cm^{-1}, and a refractive increment at constant chemical potential of solvent components of 0.1811 ± 0.0009 ml/g. The refractive increment and the absorbancy were measured as described for the antibodies. Separate calibration constants were determined for each scattering angle in order to correct for distortions in the scattering envelope. The same cell was used for all measurements. A polished polystyrene block served as a secondary standard. Corrections to scattered light for fluorescence and anisotropy were found not to be required for the calibration nor for the experimental measurements.

Aliquots of antibodies and antigens were prepared for a light-scattering experiment by chromatographing on Bio-Gel P-300 in the case of antibodies to remove the small amounts of nonspecifically aggregated antibody that form during storage and to effect equilibration with PBKS, and on Bio-Gel P-6 in the case of the antigen for the last-mentioned reason. Concentrations of freshly prepared stock solutions were measured by absorbance and mixtures of antibody and antigen, 5.00 or 5.50 × 10^{-6} M in each, were made up by gravimetric dilution. The cell was prepared by washing thoroughly with liquid detergent and rinsing repeatedly with water filtered under pressure through 0.1-μm Nuclepore filters until the intensity of light scattered by the water-containing cell was constant at the minimum observation angle (30°) and reduced to a reproducible minimum value. The cell was then dried completely by inserting carefully a small bore polyethylene tube attached to a vacuum source. The sample solutions were admitted to the cell using a pressurized filtering apparatus

which permitted aspiration of the cell contents and refiltration without handling the cell. Together the cell and filtering apparatus comprised a closed system from which losses in volume due to evaporation or transfers between vessels were eliminated. A known weight of solution was delivered into the filtering apparatus at the start of an experiment. The solution was filtered and aspirated from the cell two to three times before the intensity of scattered light was measured and then refiltered and measured again. In all cases, the reproducibility of the measurements was within the capabilities of the instrument (better than ±1.0%). A small volume of concentrated hapten (~3 mM) solution in PBKS was then delivered to the cell with a micrometer-driven syringe and the solution gently swirled, refiltered two or three times, and examined in the photometer. About ten such additions of hapten were made during a complete titration of the antibody–antigen mixture with hapten. Not less than three of the titration solutions were measured after refiltering without adding hapten. Corrections for dilution by the volume of added hapten solution (about 10% at most) were applied and it was verified that the filtration process did not remove hapten or either of the macromolecular solutes from the solution. Measurements were made at 45, 60, 75, 90, 105, 120, and 135° with respect to the incident beam and $\Delta R(0)$, the reduced intensity of forward scattered light was obtained by subtracting the fraction of light scattered by the cell filled with solvent and extrapolating the net intensity at each angle, $\Delta R(\theta)$, vs. $\sin^2(\theta/2)$ to zero. Each value of $\Delta R(0)$ has an uncertainty of ±1% and the concentrations of all solutes were known to similar accuracy.

$\Delta R(0)$ is related to the molar concentrations of nondiffusible solutes in the scattering solution by an expression obtained from one given by Casassa and Eisenberg (1964) in molar units.

$$\frac{\Delta R(0)}{1000\kappa(\Omega^c)^2} = \sum_J (\Psi_J{}^c)^2 C_J - 2\sum_J \sum_K \Psi_J{}^c \Psi_K{}^c C_J C_K B_{JK}{}^c + O(C^3) \quad (9)$$

with

$$\Omega^c \simeq \Omega^{\circ c} = \frac{\sum_J \Psi_J{}^c \Psi_J{}^{c*} C_J}{\sum_J (\Psi_J{}^c)^2 C_J} \quad (10)$$

The sums are over macromolecular components only. C_J denotes the molarity of component J, $\Psi_J{}^c$ and $\Psi_J{}^{c*}$ are the refractive increments $(\partial n/\partial C_J)_{C_3}$ and $(\partial n/\partial C_J)_{\mu_3}$, respectively, and $\Omega^{\circ c}$ is the limit obtained for Ω^c as the solution is diluted. $B_{JK}{}^c$ represents the effects of interactions between the nondiffusible components. $B_{JJ}{}^c$, where the subscripts are the same, can be identified with the virial coefficient that is obtainable from light-scattering measurements on a solution whose only macromolar solute is component J. Measured values of $B_{JJ}{}^c$ for IgG and IgG(T) antibodies are -550 ± 150 and -160 ± 170 L/mol. $B_{JJ}{}^c$ for the antigen polymers was assumed to be negligibly different from zero as PBKS at 35 °C has been reported to be an ideal solvent for PEG (Beech and Booth, 1969). The interaction coefficient between antibody and antigen, $B_{\mathrm{abag}}{}^c$, was measured in a light-scattering experiment on a mixture of normal IgG antibody (i.e., antibody with no specific affinity for the DNP group) and a fraction of antigen whose number average molar weight, $\bar{M}_n$, was 56 000 g/mol. In this system the interaction coefficient for antibody and antigen was 6800 ± 2400 L/mol. Values of $B_{\mathrm{abag}}{}^c$ for antigens of other molecular weights were calculated from this value assuming that $B_{\mathrm{abag}}{}^c$ was proportional to the molecular weight of the antigen. All $B_{JK}{}^c$ where either species J or K is a complex of antibody and antigen were estimated from $B_{\mathrm{abab}}{}^c$, $B_{\mathrm{agag}}{}^c$, and $B_{\mathrm{abag}}{}^c$ in a way described below.

Theoretical Development

Equilibrium Model. In order to interpret the data obtained from the light scattering measurements, it is necessary to develop a representation of the system by which the concentrations, C_J, of the several species of complexes consisting of antibodies, antigens, and haptens that are present in an equilibrium mixture can be expressed in terms of a small number of parameters. In any system of bivalent antigens and bivalent antibodies, only the following species of complexes are possible:

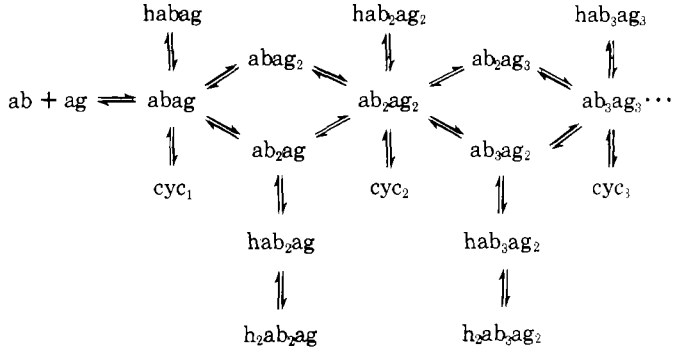
$$\mathrm{ab}_n\mathrm{ag}_n \qquad n = 1, 2, 3, \ldots$$
$$\mathrm{ab}_{n+1}\mathrm{ag}_n \qquad n = 0, 1, 2, \ldots$$
$$\mathrm{ab}_n\mathrm{ag}_{n+1} \qquad n = 0, 1, 2, \ldots$$

The number of antibodies and antigens in a complex are either the same or different by one. Complexes with equal numbers of antibodies and antigens can be either linear chains or rings; complexes with unequal numbers of antibodies and antigens can only be linear. If monovalent hapten, h, is also present in the system, additional species are possible:

$$\mathrm{hab}_n\mathrm{ag}_n \qquad n = 1, 2, 3, \ldots$$
$$\mathrm{hab}_{n+1}\mathrm{ag}_n \qquad n = 0, 1, 2, \ldots$$
$$\mathrm{h_2ab}_{n+1}\mathrm{ag}_n \qquad n = 0, 1, 2, \ldots$$

All possible reactions among the various complexes are represented in Scheme I which allows polymerization to an in-

Scheme I



definite degree. cyc_n, denotes the ring complex with n antibodies.

All of the reactions in the scheme can be assigned to one of eight classes which are listed in Table II with their equilibrium constants. The parameter K, which appears in every equilibrium constant, corresponds to the reaction of a hapten binding to an antibody site. Those equilibrium constants that involve the binding of hapten include only K and a statistical factor. K is also used as an intrinsic equilibrium constant for the formation of a bond between an antigen end and an antibody site because the portions of the antigens which interact specifically with the antibody binding sites are chemically identical with the haptens. The factor P which appears in all reactions where an antibody–antigen bond is formed in a linear complex represents those effects that, except for those attributable to differences in valence, are different for the binding of the low-molecular-weight hapten and the monovalent binding of high-molecular-weight antigen. Similarly, Q_n accounts for the

TABLE II: Equilibrium Constants for Reactions among Bivalent Antibodies, Bivalent Antigens, and Monovalent Haptens.

Reaction	Equilibrium Constant[a]
$ab_nag_n \rightleftharpoons cyc_n$	$(Q_n/2n)(V/N)K$
$ag + ab_nag_n \rightleftharpoons ab_nag_{n+1}$	PK
$ab + ab_nag_n \rightleftharpoons ab_{n+1}ag_n$	PK
$ag + ab_{n+1}ag_n \rightleftharpoons ab_{n+1}ag_{n+1}$	$4PK$
$ab + ab_nag_{n+1} \rightleftharpoons ab_{n+1}ag_{n+1}$	$4PK$
$h + ab_nag_n \rightleftharpoons hab_nag_n$	K
$h + ab_{n+1}ag_n \rightleftharpoons hab_{n+1}ag_n$	$2K$
$h + hab_{n+1}ag_n \rightleftharpoons h_2ab_{n+1}ag_n$	$(1/2)K$

[a] The units of Q are nm^{-3}; V/N is 1.66 nm^3 and P is unitless. See Crothers and Metzger (1972) for a development of the formulation of equilibrium constants for intra- and bimolecular reactions of antibodies with antigens.

constraints and nonspecific interactions which result from the cyclization of ab_nag_n to cyc_n. However, because light-scattering experiments on mixtures of antibodies with no affinity for the determinants of the antigens (Dnp groups) and antigens detected only slight interaction between these macromolecules (see above), and because of the good agreement with computations based on the models described below, values of P and Q_n different from one are taken in this system to be due entirely to configurational constraints. With this assumption P represents the fraction of configurations of the free random coil antigen which remain allowed when the antigen becomes bound at one end to a space-occupying antibody or complex. In the same way, Q_n represents the fraction of configurations accessible to the antigens and antibodies in a linear complex that remain allowed when its ends react to form a ring complex.

The simplest of the ring-forming reactions, that leading to ab=ag (cyc_1), has an equilibrium constant given by $\frac{1}{2}(V/N)QK$.[2] K enters into the equilibrium constant because a bond is formed; the factor $\frac{1}{2}$ appears, because there are two points at which the ring can open, but, once open, there is only one way it can re-form. Q is the ring closing factor. V/N is the volume per number of molecules in the standard state chosen for K and must be included because K refers to a bimolecular reaction whereas the cyclization is unimolecular. To relate Q_n, the ring closing factor for ab_nag_n to Q, Q_n is viewed as representing the probability, that the vector, $\mathbf{R}_n$, connecting the unbound antigen end in the complex ab_nag_n to the unoccupied antibody site has a magnitude within a small tolerance of zero. The linear complex ab_nag_n is a flexible chain consisting of segments which are flexible (antigens) alternating with two hinged rigid segments (the Fab arms) and so the probability density, $F(\mathbf{R}_n)$, of the end-to-end vector of the linear ab_nag_n complex may be approximately represented by a random walk in space of N steps of mean square length b^2 where N is proportional to n. The ring closure probability is then analogous to the probability that the particle undergoing the random walk returns to the origin. That is (Crothers and Metzger, 1972)

$$F(\mathbf{R}_n = 0) = \left(\frac{3}{2\pi b^2 N}\right)^{3/2} \tag{11}$$

$$\frac{Q_n}{Q} = \frac{F(\mathbf{R}_n = 0)}{F(\mathbf{R}_1 = 0)} = \left(\frac{1}{n}\right)^{3/2} \tag{12}$$

[2] For the case $n = 1$, Q_n is denoted by Q with no subscript.

The statistical factor $\frac{1}{2}$ for the formation of ab=ag becomes $1/2n$ for cyc_n which contains $2n$ bonds and thus $2n$ points at which the ring can open. The equilibrium constant for the formation of cyc_n from ab_nag_n thus is assigned the value $\frac{1}{2}(V/N)QK/n^{5/2}$. The fact that concentrations of cyclic complexes larger than ab=ag are very small (see below) makes this approximate formulation of Q_n acceptable for our particular system. With this approximation, the number of parameters needed to describe the equilibrium composition of the system is reduced to three: K, P, and Q.

With the help of the following definitions, where molar concentrations of single species are denoted by parentheses

$$[ab_nag_n] = (ab_nag_n) + (hab_nag_n)$$

$$[ab_{n+1}ag_n] = (ab_{n+1}ag_n) + (hab_{n+1}ag_n) + (h_2ab_{n+1}ag_n) \tag{13}$$

the conservation of mass equations for the system can be written.

$$AB = \sum_{n=1}^{\infty} n(cyc_n) + \sum_{n=1}^{\infty} n[ab_nag_n] + \sum_{n=1}^{\infty} n(ab_nag_{n+1}) + \sum_{n=0}^{\infty} (n+1)[ab_{n+1}ag_n]$$

$$AG = \sum_{n=1}^{\infty} n(cyc_n) + \sum_{n=1}^{\infty} n[ab_nag_n] + \sum_{n=0}^{\infty} (n+1)(ab_nag_{n+1}) + \sum_{n=1}^{\infty} n[ab_{n+1}ag_n]$$

$$H = (h) + \sum_{n=1}^{\infty} (hab_nag_n) + \sum_{n=0}^{\infty} (hab_{n+1}ag_n) + 2\sum_{n=0}^{\infty} (h_2ab_{n+1}ag_n) \tag{14}$$

AB, AG, and H represent total molar concentrations of antibody, antigen, and hapten. By making use of the following equilibrium relationships derivable from the equilibrium constants of Table II

$$[ab_nag_n] = (ab_nag_n)[1 + K(h)]$$

$$[ab_{n+1}ag_n] = (ab_{n+1}ag_n)[1 + 2K(h) + K^2(h)^2]$$

$$(ab_nag_n) = 4^nP^{(2n-1)}K^{(2n-1)}(ab)^n(ag)^n$$

$$(ab_{n+1}ag_n) = 4^nP^{2n}K^{2n}(ab)^{(n+1)}(ag)^n$$

$$(ab_nag_{n+1}) = 4^nP^{2n}K^n(ab)^n(ag)^{(n+1)}$$

$$(cyc_n) = \tfrac{1}{2}(V/N)(Q/n^{5/2})4^nP^{(2n-1)}K^{2n}(ab)^n(ag)^n \tag{15}$$

the concentration of every species can be expressed in terms of (ab), (ag), (h), K, P, and Q. To analyze the data from a light-scattering experiment, eq 9 and 14 are used with a nonlinear parameter estimating procedure described in the Appendix. The constants Ψ_J^c, Ψ_J^{c*}, and B_{JK}^c in eq 9 and 10 are calculated from the same quantities for free antibody and free antigen as follows, where N_J^{ab} and N_J^{ag} are the numbers of antibodies and antigens in the complex whose concentration is C_J.

$$\Psi_J^c = N_J^{ab}\Psi_{ab}^c + N_J^{ag}\Psi_{ag}^c$$

$$\Psi_J^{c*} = N_J^{ab}\Psi_{ab}^{c*} + N_J^{ag}\Psi_{ag}^{c*}$$

$$B_{JK}^c = N_J^{ab}N_K^{ab}B_{abab}^c + N_J^{ag}N_K^{ag}B_{agag}^c + [(N_J^{ab}N_K^{ag}) + (N_K^{ab}N_J^{ag})]B_{abag}^c \tag{16}$$

The expression for B_{JK}^c which accounts for interactions of species J with species K is obtained by counting the number

of possible interactions between the components of complexes and assuming additivity among them. This assumption derives from the fact that the second virial coefficient is proportional to the excess free energy.

The second sum on the right hand side of eq 9, when calculated by eq 16 is positive and its contribution varies from 8% for the largest antigens before any hapten is added to less than 1% for the smallest antigen in the presence of excess hapten. $\Omega^{\circ}{}_{c2}$, given by eq 10, varies from 0.86 to 0.91 for IgG antibody and from 0.93 to 1.02 for IgG(T) antibody, also depending on the size of the antigen and the amount of hapten present. These quantities cannot, consequently, be neglected but neither need they be known with high accuracy at all points in the titrations.

Estimation of Antibody Intersite Distances from Q and P. While an apparent qualitative difference between the two antibodies is clearly indicated by the values of Q determined by the experimental measurements (see Results), a quantitative interpretation of this difference in terms of average antibody hinge conformations cannot be made except in the context of a mathematical representation of the complexes. Hence, this section is devoted to developing models of varying degrees of approximation to flexible antigen–bivalent antibody complexes.

With the assumption that non-unity values of Q and P are due entirely to decreases in configurational entropy of the polymer antigens (see above) and because all the measurements were made in conditions where the polymer behaves approximately ideally, theoretical estimates of these parameters can be obtained from calculations of the relative numbers of configurations that are accessible to the polymers in the various complexes. The method used by Casassa and Tagami (1969) to calculate the fraction of configurations allowed to a random coil polymer that is confined to a void within a gel was employed. This method depends on representing the polymer by the path of a particle undergoing a random walk in space and on approximating the problem of a random walk by a boundary value problem (Chandrasekhar, 1943). For a polymer free in solution, the boundary condition is spherically symmetric and the solution is the probability density of the normal distribution. Thus for a polymer of N freely jointed segments of mean square length b^2, the probability that its end lies between $\mathbf{R}$ and $\mathbf{R} + \delta\mathbf{R}$ ($\mathbf{R} = (x,y,z)$) is $S_N(\mathbf{R})\delta\mathbf{R}$ where

$$S_N(\mathbf{R}) = \left(\frac{3}{2\pi Nb^2}\right)^{3/2} \exp\left(\frac{-3R^2}{2Nb^2}\right) \tag{17}$$

with $R^2 = \mathbf{R}\cdot\mathbf{R}$. For an ideal polymer $Nb^2 = \beta^2 M$ (Tanford, 1961, p 304), where M is the polymer molecular weight and β^2 is a constant.[3] With this substitution, eq 17 becomes

$$S_M(\mathbf{R}) = \left(\frac{3}{2\pi\beta^2 M}\right)^{3/2} \exp\left(\frac{-3R^2}{2\beta^2 M}\right) \tag{18}$$

As Q represents the fraction of configurations that are accessible to an antigen which has both ends bound to the same antibody, for a first approximation we can take the value of $S_M(\mathbf{R})$ in eq 18, for R^2 equal to $\langle R^2_{ab}\rangle$, the mean square distance between the binding sites of an antibody in the cyclic complex ab=ag, as an estimate of Q. Equation 18 as a model of the antigen configuration in a cyclic complex is least approximate for antibodies which have a fixed intersite distance (rigid hinge). A more general and less approximate model would allow for the well-established flexibility of the antibody hinge. The necessary refinement is obtained by writing

$$Q = \int S_M(\mathbf{R}) W(\mathbf{R}) \mathrm{d}\mathbf{R} \tag{19}$$

where $W(\mathbf{R})$ is the probability density of the distribution of a vector connecting the two sites of an antibody. A physical explanation that leads to eq 19 is as follows: An ab=ag complex is placed in a coordinate system such that one antibody site and one antigen end are located at the origin. Then the product of the probabilities that the second antibody binding site and the second antigen end are at the point $\mathbf{R}$ is integrated over all accessible space to obtain the total probability that the antibody intersite vector and the antigen end-to-end vector are coincident. Both $S_M(\mathbf{R})$ and $W(\mathbf{R})$ are taken to be the same as the respective probability densities of the separated molecules. In other words, both $S_M(\mathbf{R})$ and $W(\mathbf{R})$ are spherically symmetric and no account is taken at this point of the effects of space occupied by the antigen on the probability density of the antibody or vice versa (but see below). $\mathbf{W}(\mathbf{R})$ will include a Boltzmann factor which has a potential, $V(R)$, depending only on R, that is

$$W(\mathbf{R}) = \frac{\exp\left(\frac{-V(R)}{kT}\right)}{4\pi}\left[\int R^2 \exp\left(\frac{-V(R)}{kT}\right)\mathrm{d}R\right]^{-1} \tag{20}$$

where k is Boltzmann's constant and T is the absolute temperature. $V(R)$ represents the potential energy of an inter-Fab arm angle corresponding to an intersite distance of R, but, as nothing is known about it, an arbitrary specification must be made. The simplest is that for $0 \leq R \leq R_m$, where R_m is an unknown maximum value of R, $V(R)$ is constant, and $V(R) = \infty$ outside this range. With this assignment, eq 20 becomes

$$W(\mathbf{R}) = \frac{3}{4\pi {R_m}^3}, \quad 0 \leq R \leq R_m$$
$$W(\mathbf{R}) = 0, \quad R > R_m \tag{21}$$

and predicts for the uncomplexed antibody

$$\langle R_{ab}{}^2\rangle = \frac{3}{5} {R_m}^2 \tag{22}$$

With this specification, eq 19 becomes

$$Q = \left(\frac{3}{2\pi\beta^2 M}\right)^{3/2}\left(\frac{3}{{R_m}^3}\right)\int_0^{R_m} R^2 \exp\left(\frac{-3R^2}{2\beta^2 M}\right)\mathrm{d}R \tag{23}$$

where all integrations except over the radial coordinate have been done. Equations 18 and 23 yield identical values of Q at $\langle R_{ab}{}^2\rangle^{1/2} = 0$, but values which differ by 5% at an average intersite distance of 15 nm for a 50 000 molecular weight antigen.

Calculations of P from a boundary value problem representation of polymer configuration must be based on boundary values which account for perturbation of the configuration of a polymer antigen by an adjacent antibody to which it is attached at one end—an excluded volume effect. The correct boundary conditions when some space is not accessible to a polymer correspond, as DiMarzio (1965) has shown, to surfaces of zero probability positioned so as to enclose the inaccessible space. The solution to this problem is a function which is proportional to the probability density of the end-to-end vector of the polymer chain. Its integral is a normalization

[3] A value for β^2 of 0.01 nm^2 mol g^{-1} was calculated from the data in Table I and the formula $\beta^2 = 6\langle R_G{}^2\rangle/M$ (Tanford, 1961, p 304).

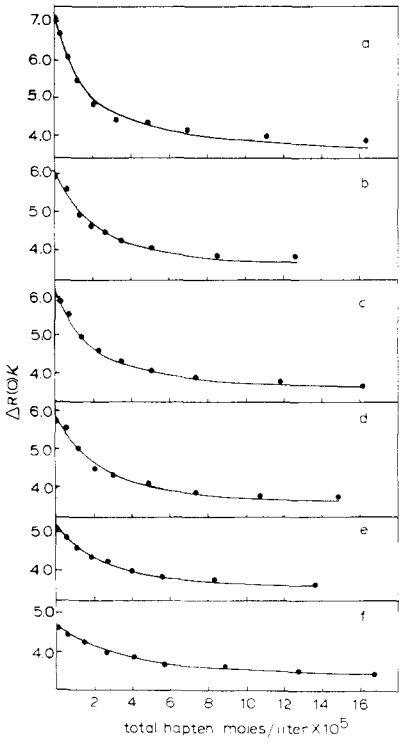


FIGURE 4: Light-scattering measurements on equimolar mixtures of IgG(T) antibody and fractions of antigens of number average molecular weights 74 800 (a), 63 000 (b), 62 200 (c), 57 800 (d), 45 400 (e), and 35 600 (f). Indicated lines are the results of nonlinear least-squares analyses.

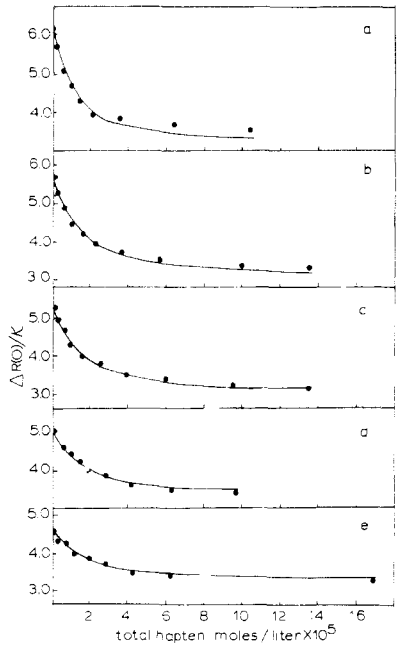


FIGURE 5: Light-scattering measurements on equimolar mixtures of IgG antibody and fractions of antigens of number average molecular weights 74 800 (a), 63 000 (b), 57 800 (c), 45 400 (d), and 35 600 (e). The indicated lines are the results of nonlinear least-squares analyses.

factor that represents the fraction of paths accessible in the presence of the barriers. Thus, the calculation of P requires that surfaces be chosen to enclose the space which is not accessible to the antibody-bound polymer. In order to maintain manageable computations, the antibody molecule is represented by a plane of infinite extent positioned at some distance, d, from the binding site to which an antigen is bound. While an infinite plane may seem an extreme simplification as a model for an antibody, it does have the essential quality of disallowing some of the polymer configurations that would intersect the antibody. A simple solution given by Carslaw and Jaeger (1959) for the corresponding problem in heat conduction is available. This solution, for a polymer with one end at the point (0,0,d) has the form

$$S_M'(\mathbf{R}) = \left(\frac{3}{2\pi\beta^2 M}\right)^{3/2}\left[\exp\left(\frac{-3}{2\pi^2 M}\right)[x^2+y^2+(z-d)^2] - \exp\left(\frac{-3}{2\beta^2 M}\right)[x^2+y^2+(z+d)^2]\right] \quad (24)$$

The integral of $S_M'(\mathbf{R})$ over all of space is a normalization factor for $S_M'(\mathbf{R})$ and an estimate of the parameter P.

$$P = \int S_M'(\mathbf{R})d\mathbf{R} = \text{erf}\left(\frac{3d^2}{2\beta^2 M}\right)^{1/2} \quad (25)$$

Equation 24 also provides estimates of Q, to be compared with measured values, which make some allowance for the asymmetry introduced into the probability density of the antigen end-to-end vector, **R**, by the antibody already bound at one end of the polymer. For the purpose of calculating Q from eq 24, an assignment for the distance of the second end of the polymer antigen (also bound to the antibody) from the barrier plane must be made because this perturbed probability density function for **R** is not symmetric with respect to the barrier plane. If both antibody sites are required to lie on a plane parallel to the barrier and radial symmetry about an axis perpendicular to the barrier plane is assumed (the barrier plane is perpendicular to the longitudinal axis of F_c), the probability density which is an estimate of Q becomes

$$Q = P^{-1}\left[1-\exp\left(\frac{6d^2}{\beta^2 M}\right)\right]\left[\frac{3}{2\pi\beta^2 M}\right]^{3/2}\left[\exp\left(\frac{-3R^2}{2\beta^2 M}\right)\right] \quad (26)$$

where P is as in eq 25.

TABLE III: Values of the Parameters Q and P for the Reaction of Bivalent Antibodies with Bivalent Antigens.

Antigen $\overline{M}_n$ (g/mol)	IgG Antibody		IgG(T) Antibody	
	Q (nm^{-3} × 10^5)	P	Q (nm^{-3} × 10^5)	P
74 800	0.68	0.27	1.21	0.41
63 000	1.09	0.36	1.70	0.37
62 200	ND	ND	1.67	0.41
57 800	1.00	0.27	1.72	0.39
45 400	0.98	0.16	2.61	0.35
35 600	1.06	0.12	2.87	0.28
25 000	ND	ND	8.64	0.18

Results

Representative data and the lines that fit them best are shown in Figures 4 and 5. In general, quite good fits of the data could be obtained from the model represented in eq 9, 14, and 15. Concentrations of the predominant species for a representative titration are shown in Figure 6. The values of Q and P obtained for all combinations of antibodies and antigens tested are listed in Table III. Two clear trends are apparent from these data. First, the value of Q decreases as the size of the antigen increases, and second, for a given antigen size, Q for IgG is smaller in all cases than Q for IgG(T). The accuracy

TABLE IV: Average Interbinding Site Distances for IgG and IgG(T) Antibodies.

Antigen $\bar{M}_n$ (g/mol)	$\langle R_{ab}^2 \rangle^{1/2}$ for IgG antibody (nm)			$\langle R_{ab}^2 \rangle^{1/2}$ for IgG(T) antibody (nm)		
	eq 18[a]	eq 23[b]	eq 26[c]	eq 18[a]	eq 23[b]	eq 26[c]
74 800	20.8	21.8	15.3	11.9	12.1	15.3
63 000	16.6	17.1	17.0	9.3	9.4	9.5
62 200				10.1	10.2	12.2
57 800	18.3	19.1	16.4	11.1	11.3	11.5
45 400	19.5	21.0	16.4	9.2	9.3	10.2
35 600	19.1	21.1	14.8	11.2	11.5	9.3

[a] Equation 18 makes no allowance for the flexibility of the antibody hinge nor for the excluded volume perturbation of the antigen configuration by the bound antibody. Averages and standard errors of the values for all antigen sizes are 18.9 ± 0.7 and 10.5 ± 0.5 nm for IgG and IgG(T), respectively. [b] Equation 23 assumes a freely flexible antibody hinge. Averages and standard errors of the values for all antigen sizes are 20.0 ± 0.9 and 10.6 ± 0.5 nm for IgG and IgG(T), respectively. [c] Equation 26 includes a correction for the perturbation of the antigen configuration by the bond antibody. Averages and standard errors of the values for all antigen sizes are 16.0 ± 0.4 and 11.3 ± 0.9 nm for IgG and IgG(T), respectively.

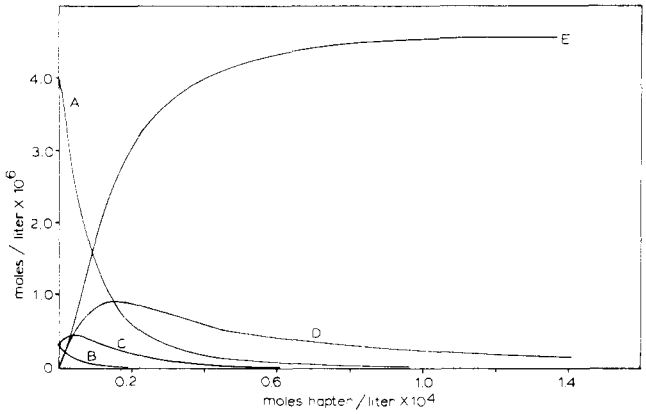


FIGURE 6: Concentrations of the predominant species in an equimolar mixture (5.00×10^{-6} moles/L) of IgG antibody and an antigen fraction of number average molecular weight 57 800. Curve A represents ab=ag, B represents all cyclic complexes larger than ab=ag, C represents all linear complexes other than h_2ab and habag, D represents habag, and E represents h_2ab.

of the values determined for P and Q could only be assessed by an empirical method because standard errors of parameters determined by a nonlinear least-squares procedure are not defined. It was determined that holding P or Q fixed at values 10% different from those giving the best fit resulted in systematic discrepancies of up to 2% between the measured and calculated data. Changing the values of P or Q by 20% increased these discrepancies to 5% at which point the quality of the fit of the data by the calculated curve had deteriorated beyond acceptable limits. Since the uncertainty in the experimental values of the scattering intensities is ±1%, a conservative assignment for the error limits of P and Q would be ±20%.

Equations 18, 23, and 26 provide three estimates of the ring closure probability, Q. The estimate provided by eq 18 takes no account of the excluded volume perturbation of the polymer configuration by the antibody to which it is bound, nor does it make any allowance for a range of antibody intersite distances as would result from a flexible hinge. The last of these two effects is included in the estimate of Q provided by eq 23 and the first is accounted for by eq 26. While it would be a simple matter to write an expression in which the refinements embodied in both eq 23 and 26 were included, the similarity of the estimates obtained by neglecting both corrections or accounting for each separately offers little incentive to do so. This similarity among the estimates does, however, provide confidence that the models do account for the major effects determining Q even though they are all simple approximations.

Estimates of the root mean square intersite distance, $\langle R_{ab}^2 \rangle^{1/2}$, for IgG and IgG(T) antibodies calculated by eq 18, 23, and 26 are listed in Table IV. Values of P required for eq 25 were taken from Table III. A best estimate of $\langle R_{ab}^2 \rangle^{1/2}$ is obtained by averaging the values obtained from the data for each antigen separately, or by treating $\langle R_{ab}^2 \rangle^{1/2}$ as an unknown parameter which is optimized by fitting all of the data for each antibody to eq 18 and 23 (Figure 6). The values of Q calculated by eq 26 with an optimized $\langle R_{ab}^2 \rangle^{1/2}$ value are also indicated. It is not possible in this case to draw a continuous curve since slightly different values of d were used for each antigen molecular weight. Optimized values of $\langle R_{ab}^2 \rangle^{1/2}$ obtained by these fitting procedures are 18.8, 18.0, and 20.3 nm for IgG and 10.6, 13.3, and 10.8 nm for IgG(T) from eq 18, 23, and 26, respectively.

The value of Q for the 25 000 molecular weight antigen is much less reliable than all the rest because of the larger uncertainty in the molecular weight of the antigen and because only a relatively small (~15%) decrease in intensity of scattered light results when a solution of complexes of antibody with this antigen is converted by the addition of hapten to one of separated antibodies and antigens. All three models predict a negative estimate of the mean square intersite distance for IgG(T) using the value of Q obtained with this antigen. For this reason, this value of Q was not included in the analysis of the values of Q by least-squares procedures to obtain estimates of $\langle R_{ab}^2 \rangle^{1/2}$. The value of Q obtained for the reaction of this antigen with IgG(T) is, however, included in Figure 7 where it is apparent that, despite its limited accuracy, it is clearly in qualitative agreement with the rest of the data.

Discussion

The consistently lower values of the ring closing factor, Q, for IgG relative to IgG(T) antibody lead to the conclusion that the decrease in configurational entropy of an antigen is less for the reaction of forming a ring with IgG(T) than for IgG antibody. Because the antigens are large and flexible and because the two types of antibodies probably have the same local binding site orientations [their light chains are thought to be identical and their heavy chains have a large degree of homology (Rockey, 1967; Weir and Porter, 1966)], the differences in the decreases in antigen configurational entropy consequent

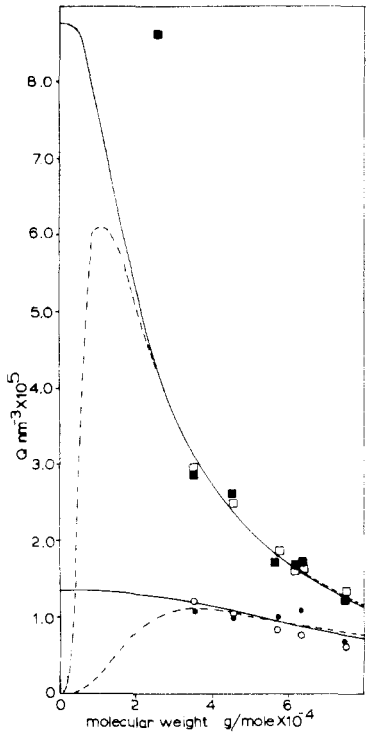


FIGURE 7: Values of Q, the ring closure probability, for the formation of cyclic complexes of bivalent antibodies with random coil bivalent antigens. The values determined for IgG(T) antibody are represented by filled squares, values determined for IgG antibody are represented by filled circles, and the results of nonlinear least-squares analyses according to eq 18, 23, and 26 are represented by the dotted curves, solid curves, and open symbols, respectively.

to forming a ring with either of these antibodies can be attributed to a difference in the average distances between the binding sites of the two antibodies. The generally good agreement between the values of the ring-closing parameter Q for the formation of the two-member cyclical complex that are deduced from the light scattering measurements and the values predicted by model representations of the antigen configuration provides substantial confidence that there are neither significant nonideal interactions in the actual system studied nor severe inadequacies in the model embodied in eq 14 and 15. On the other hand, the model is clearly not exact and there are indications of minor nonspecific interactions. Specifically, P, for both IgG and IgG(T) antibodies, decreases with antigen size whereas the model for P (eq 25) predicts the opposite dependence. Typically, the magnitude of these discrepancies is not great as is illustrated by comparing the calculated and measured values of P for the largest and smallest antigens. For IgG(T) antibody reacting with a 75 000 molecular weight antigen, P was measured to be 0.41 (Table III) corresponding to a placement of the barrier plane representing the antibody about 10 nm from the binding site. With the plane in this position, the calculated value of P for the 36 000 molecular weight antigen is 0.64 but the measured value is 0.28. This lack of agreement probably points to the existence of weak interactions between the antibodies and antigens that may be influencing the values of the parameter P and possibly also Q, or to the inadequacy in the representation of the antibody as an infinite plane (But note that theoretical estimates of P on the basis of the model enter only into eq 26). There are other obvious approximations in modeling. For example, it seems probable that the number of configurations lost to an antigen which binds to a free antibody would be less than the number lost to an antigen binding to an antibody which is part of a complex. Yet in the model assumed, the fraction of configurations remaining in both cases is P. Similar statements could be made about the configurations lost to an antigen when it becomes bound to a second antibody so that neither end is free. A fact which probably mitigates the consequences of these simplifications is that species which are composed of more than a few antibodies or antigens are at all times in small proportion (see Figure 6).

Three models of the polymer antigen in a cyclical complex with antibody were used. Essentially, the same values for $\langle R_{ab}^2 \rangle^{1/2}$ were estimated whether account was taken of the perturbation on the antigen configurations by the bound antibody or not (eq 26) or whether the integrated model that accounts for the distribution in antibody intersite distances (eq 23) was used. It may therefore be concluded that even these principal perturbations have little practical consequence in this instance, and that the values of $\langle R_{ab}^2 \rangle^{1/2}$ obtained for IgG antibody which are rather larger than would be expected from estimates of dimensions of antibody molecules are probably indicative of minor systematic errors in all values of Q, in the models used to estimate $\langle R_{ab}^2 \rangle^{1/2}$, or in the polymer parameter β^2.

Estimates of the length of the Fab arms range from 6.5 nm (Valentine and Green, 1967) obtained by electron microscopy to nearly 10 nm (Pilz et al., 1970) determined by measurement of low angle x-ray scattering by solutions of antibodies and antibody fragments. On the basis of the larger of these dimensions, our data would suggest that IgG molecules are essentially T shaped in solution and IgG(T) molecules have an inter-Fab arm angle of about 60°. While this is a more quantitative conclusion than our measurements allow, it is consistent with the findings of other approaches to the problem of determining the shape of IgG in solution (Pilz et al., 1970; Werner et al., 1972). If the estimates of $\langle R_{ab}^2 \rangle^{1/2}$ for both antibodies carry similar errors in the same direction, then 60° would be an upper limit on the average inter-Fab arm angle in IgG(T) or, in other words, IgG(T) would have a fairly compact conformation.

Finally, it must be added that our experiments do not provide any direct information of the flexibility of the hinge. Only estimates of average intersite distances are obtained. But they do, of course, give insight into limitations on the extensibility of the hinge. Studies similar to this one in which rigid antigens with known distances between determinants are used might provide estimates of flexibility by allowing a determination of maximum and minimum intersite distances. Measurements of ring complex formation with smaller flexible antigens might also be useful (see Figure 7); however, with smaller antigens, the extent of complex formation could not be monitored by light scattering.

In conclusion, the measurements in this report and those in the previous paper (Archer and Krakauer, 1977), which indicate that there are fluorescent residues in IgG(T) antibodies but not in IgG antibodies, which can transfer excitation energy to a hapten bound at either site of the antibody, demonstrate that the Fab arms are closer together in IgG(T) than in IgG antibodies, as suggested by Klinman and Karush (1967). A conformational basis for the different abilities of IgG and IgG(T) antibodies to precipitate antigens has thus been established.

Acknowledgments

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Appendix

The model which represents the equilibrium state of a mixture of bivalent antibodies and antigens is embodied in the conservation of mass statements (eq 14) which relate the concentration of every species to the parameters K, Q, P, and to the concentrations of free antigen, free antibody, and free hapten. The conservation of mass equations can be solved for the free antibody concentration in terms of other quantities. The remaining system of equations which must be solved for each mixture can then be written as

$$\begin{aligned} \mathrm{Ag}_i - f_{1i}(\mathrm{ag}_i, h_i, K, Q, P) &= 0 \\ H_i - f_{2i}(\mathrm{ag}_i, h_i, K, P) &= 0 \\ \Delta R(0)_i - f_{3i}(\mathrm{ag}_i, h_i, K, Q, P) &= 0 \end{aligned} \qquad (1A)$$

where Ag_i, H_i, and $\Delta R(0)_i$ denote the known total antigen, total hapten, and measured intensity of scattered light for the ith solution of antibody, antigen, and hapten. The lower case symbols denote the free concentrations of antigen and hapten, and f_{1i}, f_{2i}, and f_{3i} are quantities computed from these last by means of eq 9 and 14. For the measurement made on a mixture of antibody and antigen with no hapten, the equation which involves H_i does not apply. Thus for a series of m measurements (m different total concentrations of hapten, H_i) on solutions of a particular antibody–antigen pair, $3m - 1$ equations must be solved for $2m + 1$ unknowns. The unknowns include Q, P, m values of ag_i, and $m - 1$ values of h_i. Because these equations are nonlinear and include infinite sums which cannot be expressed in closed form, numerical procedures were necessitated in order to interpret the data. The problem was solved using an implementation of Marquardt's algorithm (1963) developed by Fred T. Krogh at the Jet Propulsion Laboratory, Pasadena, Calif. The program uses a nonlinear least-squares approach, choosing parameter values to minimize

$$\rho^2 = W^2 \sum_{i=1}^{m} (\mathrm{Ag}_i - f_{1i})^2 + W^2 \sum_{i=2}^{m} (H_i - f_{2i})^2 + \sum_{i=1}^{m} (\Delta R(0)_i - f_{3i}) \qquad (2A)$$

W, a factor which weights the contributions of the conservation of mass equations to ρ^2 was chosen to be 10^4. With this weighting, all parameters ag_i and h_i were determined to a relative accuracy of 0.1% or less. Thus, although each problem had a large number of parameters (up to 21), since the $2m - 1$ value of ag_i and h_i were completely determined by Q and P, only these last two remained adjustable for the purpose of fitting the data. The uniqueness of the values of Q and P which produced the best fit was substantiated by verifying that the program would converge to the same solution from several different starting points. It was also verified that the values of Q and P were not sensitive to small experimental errors in the data. "Noise" of magnitudes up to 3% introduced into the data caused only minor changes of 15% or less in the values of Q and P.

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