Interpretation of Fluorescence Quenching Titrations of Antihapten Antibody Having Overlapping Hapten Quenching Domains†

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ABSTRACT: Anti-dinitrophenyl antibody of the equine IgG(T) class, fractionated to very near homogeneity in affinity for the hapten, was found, nevertheless, to exhibit disproportionately high initial quenching of its fluorescence on titration with the hapten. This behavior is satisfactorily interpretable if allowance is made for the greater quenching of the fluorescence of the antibody molecule by the first hapten molecule binding than the second. Procedures are presented to permit the evaluation of the two quenching parameters, whose ratio varied from 1.5 to 2.0 in the various fractions of antibody examined, and the calculation, by these means, of binding isotherms. These were found to be in close agreement with isotherms calculated from measurements of the reduction of the absorbance of the hapten on being bound to the antibody. The applicability of the procedures for the evaluation of fluorescence quenching titrations is limited by the requirement of homogeneity in the quenching parameters for the two sites, but not significantly by any requirement for homogeneity in affinity for ligand in a population of antibody molecules.

The quenching of intrinsic antibody fluorescence by bound hapten, first described by Velick *et al.* (1960), is commonly used to monitor titrations of antibodies to determine binding isotherms for a variety of haptens. To interpret these titrations, the assumption is made that reductions in fluorescence which occur as hapten is added to antibody are proportional to the fraction of antibody sites occupied. This assumption is made despite the rather frequent observation that the extent of quenching at low extents of binding exceeds the fractional saturation even if all added hapten is assumed bound (Parker, 1967; Kabat, 1968). McGuigan and Eisen (1968) have shown that heterogeneity in the affinity of binding sites for the dinitrophenyl hapten is accompanied by heterogeneity in antibody fluorescence and in susceptibility to quenching, and suggest that heterogeneity in these spectral properties can account for the obvious lack of proportionality between hapten binding and fluorescence quenching. These authors also concluded that overlapping of domains quenched by the two haptens bound to an antibody molecule could not account for the initial excess quenching as it was also noticed, although to a lesser degree, in titrations of monovalent fragments.

Preliminary to an investigation of the flexibility of the hinge region in equine antibody of the IgG(T) class (Weir *et al.*, 1966) with synthetic divalent antigens bearing dinitrophenyl determinants, we undertook to obtain a thermodynamic characterization of the binding of monovalent hapten to this antibody. It became evident that the assumption of proportionality between extent of quenching and extent of binding of hapten to IgG(T) was incorrect. We present below procedures that permitted, by allowing for energy transfer within the antibody molecule to either binding site, the successful application of the fluorescence quenching method in our study, procedures that can be applied to any antibody–hapten system which is reasonably homogeneous, and that provide parameters having potential structural implications.

Experimental Procedures

Antibody. A shetland pony that had been inoculated repeatedly with N_2Ph[1] bovine γ globulin for 2 years was bled several times within a 3-week period to obtain 6 l. of serum containing anti-dinitrophenyl antibody. Specific antibody was isolated essentially in the manner described by Eisen (1964) except that an affinity column of ϵ-N_2Ph-lysyl–agarose (Wofsy and Burr, 1969) was substituted for the dinitrophenylated proteins. Because the capacity of the affinity column to adsorb antibody was roughly 0.1 of the total amount of antibody in the serum, the isolation procedure also achieved fractionation of the antibody with respect to affinity for hapten. The entire volume of serum was passed through the column and adsorbed antibody eluted six times, each cycle yielding about 2 g of antibody. Antibody removed from the serum on the first, fifth, and sixth passes through the column (fractions 1, 5, and 6, respectively) was studied. Over 90% of the specific antibody was found to be of the IgG(T) class and was separated from other classes by DEAE-cellulose chromatography (Klinman and Karush, 1967). Absence of other immunoglobulin classes was verified by immunoelectrophoresis and immunodiffusion in agar against monospecific antisera to IgG, IgM, IgA, and aggregating immunoglobulin (McGuire and Crawford, 1972). Molar concentrations of antibody and Fab fragments were calculated on the basis of the following absorbancies at 279 nm (Rockey, 1967) and molecular weights (Montgomery *et al.*, 1969): for IgG(T), $E^{g/l}_{279} = 1.47$, mol wt = 152,000; for Fab fragments, $E^{g/l}_{279} = 1.46$, mol wt = 50,000. Concentrations were verified by spectrophotometric titrations described

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[1] Abbreviations used are: N_2Ph, 2,4-dinitrophenyl; N_3Ph, 2,4,6-trinitrophenyl.

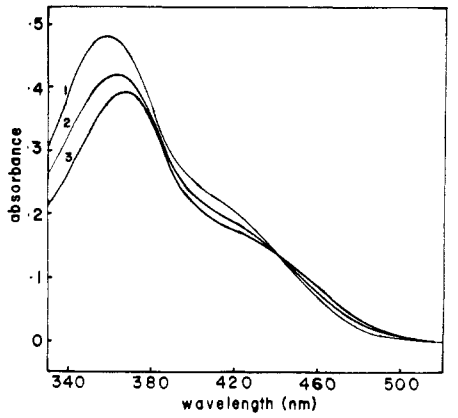

FIGURE 1: The absorption spectrum of N_2Ph-glycine in the presence of fraction 6 antibody. The concentration of N_2Ph-glycine is 3.2×10^{-5} M for all spectra. Antibody concentrations are 0, 1.17, and 2.96 mg/ml for curves 1, 2, and 3, respectively. All spectra were measured in a double beam spectrophotometer whose reference cell contained a solution identical with that in the sample, but lacking only hapten.

TABLE I: Results of Spectrophotometric Titrations on Concentrated Solutions.[a]

Antibody Fraction	Site Purity[b]	$\epsilon_h - \epsilon_{hs}$ (M^{-1} cm^{-1})
1	1.05 ± 0.05 (0.02)	3730 ± 380 (130)
5	[c]	4280 ± 90 (70)[c]
6	0.98 ± 0.06 (0.02)	4890 ± 550 (180)
5 Fab	1.02	2580

[a] These results are a summary of ten titrations of fraction 1, two of fraction 5, nine of fraction 6, and one of fraction 5 Fab. Listed uncertainties are standard deviations and, in parentheses, standard errors of means. [b] Site purity is the quotient of site concentration determined by the titration and that determined by absorbance at 279 nm. [c] $\epsilon_h - \epsilon_{hs}$ for fraction 5 was estimated from initial slopes of titrations of dilute solutions.

below. All experiments were carried out on solutions of antibodies containing 0.15 M sodium chloride and 0.01 M sodium phosphate at pH 7.80.

Digestion of specific IgG(T) antibody according to the method of Porter (1959) produced divalent fragments. These were allowed to react with 0.1 M 2-mercaptoethanol overnight and alkylated with *N*-ethylmaleimide to produce monovalent Fab fragments (Weir and Porter, 1966). The monovalent fragments were freed of inactive digestion products by chromatography on DEAE-cellulose, Bio-Gel P-100, and the N_2Ph–agarose immunoadsorbant. Single symmetrical peaks were observed in photographs of schlieren patterns taken during ultracentrifugation of both mono- and divalent fragments. The sedimentation coefficients were essentially those reported in the literature, 5 S for divalent and 3.5 S for monovalent fragments. Recoveries from the digestive and reductive steps were from 10 to 20%.

Hapten. N_2Ph-glycine was used as purchased. Concentrations were calculated from a molar absorbancy of 15,900 M^{-1} cm^{-1} at 360 nm (Little and Donahue, 1968) and 11,600 M^{-1} cm^{-1} at 340 nm, the latter value determined from the ratio of absorbances at the two wavelengths.

Spectrophotometric Titrations. The large reduction in absorbancy of N_2Ph-glycine at 340 nm consequent to binding to antibody (Warner and Schumaker, 1970) was used to monitor titrations of concentrated solutions of antibodies (1–4 mg/ml) from which site purity was determined, and of dilute solutions of antibodies (~0.1 mg/ml) from which binding isotherms were calculated. Titrations of concentrated solutions were performed by adding N_2Ph-glycine to a measured volume of antibody solution and measuring the absorbance after each addition. Because these solutions often became turbid after the first few additions, a different protocol was also used in which the absorbances of separate solutions containing identical concentrations of antibody and increasing concentrations of N_2Ph-glycine were measured. As the presence of an isosbestic point was verified (Figure 1), the difference in absorbance (ΔA) due to hapten in a solution of hapten alone and one with antibody and the same total concentration of hapten may be written as

$$\Delta A = (\epsilon_h - \epsilon_{hs})[hs] \quad (1)$$

where ϵ_h and ϵ_{hs} represent the molar absorbancies of free and bound hapten and [hs] is the molar concentration of hapten–antibody site complex. A plot of ΔA *vs.* total hapten concentration can be fitted by two straight line segments with the value of the abscissa at their intersection a measure of total site concentration.

Spectrophotometric titrations of dilute solutions of antibody employed 10-cm path-length cuvets and measurements of absorbance were made with Cary Model 14 or 15 spectrophotometers. The extent of binding, θ, was determined by

$$\theta = \frac{[hs]}{[s_t]} = \frac{\Delta A}{\Delta A_\infty} \quad (2)$$

where $[s_t]$ is the total site concentration, and ΔA_∞ is the value of ΔA when all sites are occupied by hapten. All measurements were made in air conditioned rooms where the ambient temperature was 22–25°.

Fluorometric Titrations. All measurements of fluorescence were made with a modified Brice Phoenix light scattering photometer. The exciting light was defined by a HA85UV mercury lamp and an interference filter having a peak transmittance at 290 nm. Pyrex glass placed at the entrance aperture of the photomultiplier (EMI9781) detector housing served to cut off scattered and reflected light. In titrations of dilute solutions of antibody, the detector was positioned to receive light emitted at 90° to the exciting beam. In a titration of a concentrated solution of antibody, fluorescence was measured at the solution surface first irradiated by the exciting light to lessen the effects of the high concentration on fluorescence measurements (Udenfriend, 1962). In this case the detector was positioned so that its optical axis made an angle of 30° with the axis of exciting light. Corrections for nonspecific quenching and dilution were applied. Blank fluorescence intensities were less than 5% of the initial sample values. The cell table was maintained at 25°.

Results

Spectrophotometric Titrations. Results of titrations of concentrated solutions are summarized in Table I. The appearance of turbidity during some of these titrations had no detectable

effect on estimates of site purity as results by both methods described above were not significantly different.

Data obtained from spectrophotometric titrations of dilute antibody solutions were interpreted by assuming a Sips distribution (Sips, 1948) of binding free energies. Values for k_0, the average association constant, and a, the index of heterogeneity, were determined by the parameters of a straight line fitted to a plot of ln $\theta/(1-\theta)$ *vs.* ln [h] according to the equation (Karush, 1962)

$$\ln \theta/(1-\theta) = a \ln [h] + a \ln k_0 \quad (3)$$

where [h] is the molar concentration of free hapten. A summary of the values obtained for ln k_0 and a is presented in Table II. It is important to note here that for intact antibody, values for a indicate near homogeneity but the value of a for the monovalent fragments indicates marked heterogeneity. The values of ln k_0 for fractions 1, 5, and 6 correspond to values of k_0 of 14.5, 3.37, and 2.17 $\times$ 10^6, respectively. Over 80% of the total antibody originally in the serum had affinities within the range spanned by fractions 1 and 6. Allowing for heterogeneity within each fraction, a likely estimate for the value of Sips a for the unfractionated antibody is about 0.7.

Fluorometric Titrations. Because adoption of the usual assumption of proportionality between extents of binding and quenching led to the severe inconsistencies mentioned in the introductory statement, an alternate treatment which allows for unequal quenching by the first and second haptens binding to an antibody molecule was used. An expression for the measured fluorescence, F, is given by

$$F = F_0\frac{[ab]}{[ab_t]} + (1-q_1)F_0\frac{[hab]}{[ab_t]} + (1-q_1-q_2)F_0\frac{[h_2ab]}{[ab_t]} \quad (4)$$

where F_0 is the initial fluorescence, q_1 and q_2 are the reductions in quantum yield of an antibody molecule when the first and second sites become occupied by hapten, and [ab_t], [ab], [hab], and [h_2ab] are molar concentrations of total, unbound, singly, and doubly bound antibody, respectively. Because the spectrophotometric titrations indicated the antibody preparations to be nearly homogeneous, they were assumed to be so to facilitate interpretation of the fluorescence measurements. From the expressions for the equilibrium constants for the two reactions occurring

$$h + ab \rightleftharpoons hab \qquad k' = 2k = [hab]/[h][ab] \quad (5a)$$

$$hab + h \rightleftharpoons h_2ab \qquad k'' = {}^1/_2k = [h_2ab]/[h][hab] \quad (5b)$$

where k is an intrinsic site binding constant and the factors 2 and $^1/_2$ result from statistical considerations (Tanford, 1961), an equation for the conservation of mass, and the definition of θ in terms of the species of interest (eq 6)

$$\theta = \frac{[hab] + 2[h_2ab]}{2[ab_t]} \quad (6)$$

we can derive the following

$$[ab]/[ab_t] = (1-\theta)^2 \quad (7a)$$

$$[hab]/[ab_t] = 2\theta(1-\theta) \quad (7b)$$

$$[h_2ab]/[ab_t] = \theta^2 \quad (7c)$$

TABLE II: Summary of Results from Binding Isotherms Obtained by Spectrophotometry.

Antibody Fraction	ln k_0	a
1	16.49 ± 2.60 (0.55)[a]	0.99 ± 0.11 (0.02)[a]
5	15.03 ± 0.59 (0.10)	0.78 ± 0.02 (0.01)
6	14.59 ± 0.71 (0.13)	0.85 ± 0.03 (0.01)
5 Fab	13.76 ± 1.38 (0.40)	0.55 ± 0.04 (0.01)

[a] Listed uncertainties are standard deviations and standard errors of the means.

Upon substitution of eq 7a–c and the definition of observed quenching, $Q = (F_0 - F)/F_0$, into eq 4 and solving for θ, we obtain

$$\theta = \frac{q_1 - [q_1^2 - Qq_1 + Qq_2]^{1/2}}{q_1 - q_2} \quad (8)$$

A rearrangement of eq 8 which is useful for determining q_1 and q_2 is

$$Q/\theta = 2q_1 - (q_1 - q_2)\theta \quad (9)$$

If the assumption that $a = 1.0$ is not made, the expression corresponding to eq 9 is

$$Q/\theta = 2q_1 - (q_1 - q_2) \times \left\{\frac{4^a + 2(4-4^a)\theta - [4^{2a} + 4 \times 4^a(4-4^a)\theta(1-\theta)]^{1/2}}{2(4-4^a)\theta}\right\} \quad (9a)$$

If an antibody concentration is chosen such that most of the hapten added in the initial stages is bound and fractions of total hapten that remain free are large enough to determine k without loss of precision in the middle range of the titration,[2] q_1 and q_2 can be determined from the same titration used to estimate ln k_0 and a in the following way. By guessing a value for k which can be very approximate, θ in the initial part of the titration can be estimated. At the end of the titration, θ tends to 1.0 as Q/θ approaches a constant value ($q_1 + q_2$). In this way, the line Q/θ *vs.* θ is defined by values of θ near zero and one. Values of q_1 and q_2 are obtained from the parameters of a straight line fitted to these points according to eq 9. Once q_1 and q_2 have been assigned, θ for the rest of the titration is calculated by eq 8 and a logarithmic Sips plot is made from which values for k_0 and a are determined. These values for k_0 and a can be used to refine the estimates of θ near zero and one to improve the assignments of q_1 and q_2. The process can be repeated if necessary until self-consistent results are obtained. Results of these procedures are given in Tables III and IV along the results obtained if binding and quenching are assumed proportional. Values of ln k_0 listed in Table III for fractions 1, 5, and 6 correspond to values of k_0, if q_1 is not assumed equal to q_2, of 22.3, 2.84, and 2.57 $\times$ 10^6, and, if q_1 is assumed equal to q_2, of 272, 13.9, and 8.2 $\times$ 10^6, respectively. The values of a and k_0 obtained when q_1 and q_2 are

[2] An appropriate concentration is one for which the product of the site association constant and total site concentration is about 5–10.

TABLE III: Summary of Results from Binding Isotherms Obtained by Fluorometry.[a]

Antibody Fraction	$q_1 \neq q_2$		$q_1 = q_2$	
	ln k_0	a	ln k_0	a
1	16.92 ± 0.97 (0.21)	1.00 ± 0.04 (0.01)	19.42 ± 2.67 (0.95)	0.48 ± 0.05 (0.02)
5	14.86 ± 0.50 (0.09)	0.80 ± 0.02 (0.01)	16.45 ± 0.87 (0.18)	0.51 ± 0.02 (0.01)
6	14.76 ± 0.25 (0.06)	0.92 ± 0.01 (0.004)	15.92 ± 0.74 (0.21)	0.65 ± 0.02 (0.01)
5 Fab			15.89 ± 1.83 (0.65)	0.55 ± 0.05 (0.02)

[a] Listed uncertainties are standard deviations and standard errors of the means.

assumed equal indicate marked heterogeneity, comparable to that frequently reported for antihapten antibody but clearly at variance with that calculated from the spectrophotometric titrations. Equally clearly, the concordance is quite satisfactory if q_1 and q_2, calculated by means of eq 9, are used. Figure 2 presents a comparison of the isotherms calculated from titrations monitored by spectrophotometry and by fluorometry, the latter on the basis of the two alternative assumptions about q_1 and q_2. The absence of data points corresponding to values of $\theta < 0.4$, if θ is taken to be just proportional to the observed quenching ($q_1 = q_2$), is due to the fact that the apparent excessive initial quenching leads to calculated values of free hapten less than zero. Results of this type are also commonly encountered in the literature.

Fluorescence quenching titrations of antibody solutions concentrated enough that hapten binding was essentially stoichiometric were performed to relate binding to quenching over the entire titration range independently of the parameters k_0 and a. A plot of Q/θ *vs.* θ for a titration of this type is shown in Figure 3. In this titration fluorescence was measured at the front surface of the irradiated solution to lessen the effects of concentration on the measurements. However, because the absorbance due to the hapten at equivalence was about 0.35 cm^{-1} with respect to emitted light and about 0.14 cm^{-1} with respect to exciting light, the inner filter effects (Parker and Barnes, 1957) were marked. Corrections for the nonspecific quenching of fluorescence by hapten due to its absorbance of incident and emitted light were calculated and found to be greatest, per mole of hapten added, at the start of the titration and to decrease as hapten concentration increased. However, the actual correction applied to the data was the conventional linear one based on the final slope of a plot of fluorescence against hapten concentration. This correction was therefore inadequate in the early part of the titration, leading to greater apparent specific quenching by hapten and an overestimate of q_1. The derived values were $q_1 = 0.53$ and $q_2 = 0.13$.

Because the demonstration that the quenching of the fluorescence of monovalent Fab fragments by hapten was indeed proportional to the extent of the binding of the hapten would have provided final verification that the nonlinear pattern of quenching of the fluorescence of divalent antibody was due to the influence of the first hapten to bind on the quenching effect of the second, a fluorescence quenching titration was performed on monovalent fragments. The Fab fragments titrated were proved to be free of inactive material by a spectrophotometric titration (Table I) but also to be very heterogeneous in affinity (Table II). A maximum corrected quenching of only 0.74 and more quenching than could be accounted for by binding up to values of $\theta = 0.5$ were observed. Fractionation of these fragments by the procedure used for the complete antibody was attempted but could not be satisfactorily performed because an insufficient amount of material, about 10 mg, was available, and because the procedure is less

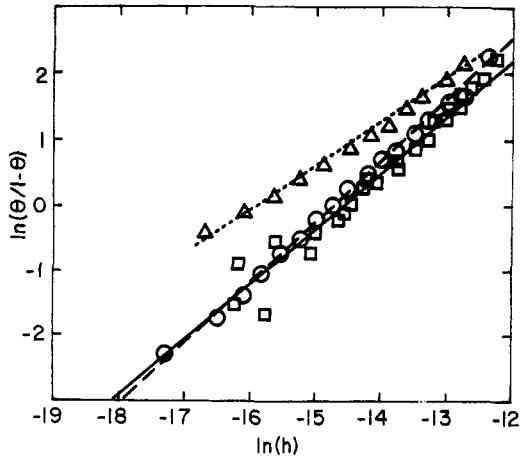

FIGURE 2: Representative binding data for fraction 6 antibody: (□) by spectrophotometry; (○) by fluorometry with $q_1 \neq q_2$; and (△) by fluorometry with $q_1 = q_2$. The indicated lines are least-squares fits of the data.

TABLE IV: Reduction of Antibody Fluorescence by Bound Hapten.[a]

Antibody Fraction	q_1	q_2	$q_1/(q_1 + q_2)$[b]
1	0.385 ± 0.005 (0.002)	0.251 ± 0.021 (0.009)	0.605
5	0.453 ± 0.007 (0.003)	0.230 ± 0.031 (0.013)	0.663
6	0.423 ± 0.004 (0.002)	0.212 ± 0.020 (0.008)	0.667

[a] q_1 and q_2 are the reductions in fluorescence of an antibody molecule which occur when the first and second haptens, respectively, bind. The listed uncertainties are standard deviations and standard errors of the means. [b] Fractional quenching due to the binding of a hapten to one site of a free antibody molecule. $q_2/(q_1 + q_2) = 1 - q_1/(q_1 + q_2)$ = fractional quenching due to binding to the second site, the first being occupied.

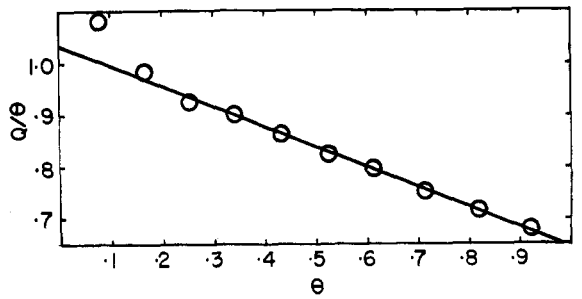

FIGURE 3: Results of a fluorescence quenching titration of fraction 5 antibody at high concentration, 1.96 mg/ml. Fluorescence was measured at the front surface of the solution.

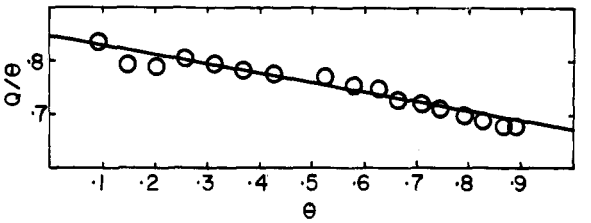

FIGURE 4: Results of a fluorescence quenching titration of fraction 6 antibody at low concentration. k_0 and a, used to calculate θ, were obtained from a spectrophotometric titration. The line is a least-squares fit of the indicated points. Derived values for q_1 and q_2 are $q_1 = 0.423$ and $q_1 = 0.250$.

effective with monovalent fragments than with divalent antibody where, because of the possibility of binding with both sites to the immunoadsorbant, ratios of affinity constants (k) are effectively squared and differences in affinity thereby magnified.

Discussion

While the differences we have found in $\epsilon_h - \epsilon_{hs}$ and ln k_0, as well as in $\Delta H°$, to be reported separately, for the different fractions of IgG(T) confirm the molecular heterogeneity of specific antibody, the presence of an isosbestic point and values of a near 1.0 indicate that the degree of fractionation achieved by our preparative procedure was substantial. According to the Sips distribution, even antibody of fraction 5 which had the smallest value of a ($a = 0.8$) was heterogeneous only to the extent that 80% of the binding sites had a binding constant within a factor of 4 of k_0. (The greater heterogeneity of fraction 5 over the other fractions may be due to its having been stored about 3 months after fractionation before being studied. Fractions 1 and 6 were used shortly after fractionation.) Because our measurements were made on antibody fractions demonstrated to be nearly homogeneous both in affinity for hapten and in a spectral property, the disproportionality between quenching and hapten binding that we observed cannot be explained by molecular heterogeneity. The close agreement of the values for ln k_0 and a obtained by spectrophotometry which reflects the properties of the bound hapten, and by fluorometry which reflects the properties of the antibody, when allowance is made for the inequality of quenching of the two sites, combined with the discrepancies between these values when q_1 is assumed equal to q_2, lead us to conclude that unequal quenching was in fact occurring in the N_2Ph-glycine–IgG(T) system. This phenomenon may be the result of an overlap of the quenching domains of the haptens at the two sites or to an induction of a conformational change. We lean toward the former interpretation because it is difficult to conceive a conformational change, consequent to the binding of the first hapten, that would have the observed disproportionate effect on the fluorescence of the antibody and yet not alter the affinity of the second site for hapten, the latter also a conclusion supported by our data.

In Figure 4 we have plotted Q/θ *vs.* θ for a titration of fraction 6 antibody where θ was calculated from the values of k_0 and a determined from a spectrophotometric titration. The fit of the calculated points to a straight line from which derived values for q_1 and q_2 which agree well with values obtained solely from fluorometry supports the correctness of our treatment.

The fluorescence titration of a concentrated solution of fraction 5 antibody also indicated that q_1 is not equal to q_2 for IgG(T). The discrepancy between the values obtained for q_1 and q_2 by titrations of concentrated and dilute solutions can be attributed to the nonlinear nonspecific quenching of fluorescence in concentrated solutions (Parker and Barnes, 1957).

The attempt to further verify the overlap of hapten quenching domains in IgG(T) by demonstrating the expected proportionality between hapten binding to and fluorescence quenching of monovalent fragments was not successful. The excess initial quenching and low maximum quenching observed in a titration of these fragments (see Results) must reflect structural heterogeneity introduced by the digestion and reduction and alkylation required for their preparation, all rather destructive procedures as evidenced by the low final recoveries. This heterogeneity was not present in the parent molecules. The relatively small decrease in absorbancy of haptens bound to these fragments (Table I) is also indicative of structural alterations as is the heterogeneity in affinity of these fragments which is revealed by the low value of a in a spectrophotometric titration (Table II). An attempt to fractionate these fragments by the procedure employed for intact antibody failed probably for the reasons already given.

One noteworthy property of IgG(T) is its nonprecipitability with many antigens. This, together with other behavioral properties, has prompted the suggestion (Klinman and Karush, 1967) that the Fab arms of IgG(T) may be positioned closer together on the average than in IgG. If this speculation is correct, there is a basis in structure for q_1 not being equal to

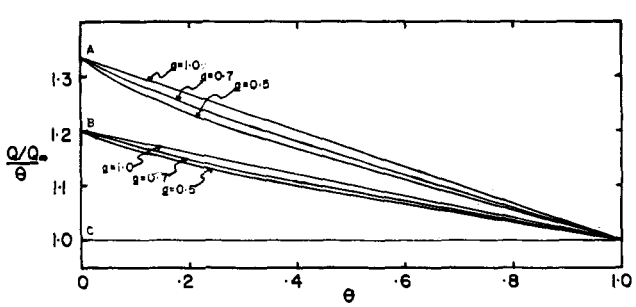

FIGURE 5: The dependence of relative molecular quenching on the degree of saturation for antibody populations homogeneous in q_1 and q_2 but heterogeneous in affinity. Q was normalized by dividing by maximum quenching, $Q_\infty = q_1 + q_2$. For $a = 1.0$, the curves were calculated from eq 9; for $a \neq 1.0$, the curves were calculated from eq 9a. The family of curves labeled A was calculated for $q_1 = 2q_2$; those labeled B were calculated for $q_1 = 1.5q_2$; the horizontal line labeled C was calculated for $q_1 = q_2$.

q_2 in IgG(T) which may be lacking in IgG. However, the occurrence of "anomalous" excess quenching in titrations of IgG (Parker, 1967; Kabat, 1968) makes it likely that q_1 is not always equal to q_2 for this molecule either. Furthermore, Little and Eisen (1968) found that divalent Fab fragments from rabbit IgG antibody specific for N_2Ph and N_3Ph haptens were quenched more completely than monovalent fragments derived from the same antibodies. A probable explanation of these observations is that there are amino acid side chains in some IgG's which can transfer excitation energy to a hapten bound at either site.

The assumption of homogeneity permits the derivation of the comparatively simple equation 8. A more cumbersome expression can be derived to relate the observed quenching to the extent of binding without making this assumption (9a). The assumption is, however, necessary for another reason. As McGuigan and Eisen have shown (1968), heterogeneity in affinity reflects heterogeneity in structural and spectral properties. In a heterogeneous population of antibodies, it is not possible to separate unequal quenching due to structural differences among antibodies from unequal quenching due to differences in quenching by the first and second hapten molecules binding. If, however, it can be established that it is a good approximation to assume single values of q_1 and q_2 for an entire population of antibodies, eq 8 can be used to calculate θ for Q, q_1, and q_2 with relatively little error even in the presence of heterogeneity in affinity. In Figure 5, we have plotted the relative molecular quenching, $Q/Q_\infty\theta$, *vs.* θ, assuming homogeneity in q_1 and q_2, but allowing for heterogeneity in affinity. It is evident that using eq 8, which is equivalent to assuming $a = 1.0$, will introduce a much smaller error than neglecting a difference between q_1 and q_2.

The method presented in this paper is applicable to any antibody–hapten system which is not too heterogeneous. The requirement of near homogeneity may be satisfied by the relatively simple fractionation procedure described herein, or by others, for example that used by Eisen and Siskind (1964).

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