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The Shape of Immunoglobulin G Molecules in Solution

(rabbit antiserum/hapten/fluorescein/singlet–singlet energy transfer/conformation)

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ABSTRACT **We have studied the shape of rabbit Immunoglobulin G molecules in solution by using singlet–singlet energy transfer to determine the minimum distance between the two hapten binding sites. A hybrid antibody was prepared in which one site specifically bound the energy donor, ε-dansyl-lysine, and the other site bound the energy acceptor, fluorescein. For this donor–acceptor pair, R_0 was calculated to be 4.8 ± 0.2 nm (48 ± 2 Å). From a comparison of the lifetime of the donor's excited state in the presence or absence of acceptor, it was found that no energy transfer had occurred in the hybrid. Since the maximum distance over which transfer is measurable was 8.2 nm (82 Å; 1.7 R_0), and since the Fab moieties exhibit segmental flexibility, the average distance between the two hapten-binding sites was estimated to be 9.2–10 nm (92–102 Å). If one assumes that the length of the Fab fragment is 7 nm (70 Å), the corresponding minimum angle between Fab moieties, α_M, would be 80–95°. The molecules in solution, thus, have an open *Y*- or *T*-shaped configuration in which the hapten binding sites are not more than 2.5 nm (25 Å) from the extreme ends of the Fab fragments. The existence of conformations in which α_M is less than 80°, as has been observed in some antibody–antigen complexes, must therefore be the result of definite conformational changes.**

Although the conformation of IgG molecules has been studied by several techniques, the spatial relationship of the Fab and Fc fragments in solution has not been defined. The results of electron microscopy, transient electric birefringence, and x-ray scattering studies have suggested that the IgG molecule is probably *Y*- or T-shaped (1–5) with the binding sites located at the ends of the Fab fragments (2, 5). However, it is not known whether there is a preferred angle between the Fab fragments in the free immunoglobulin in solution. If such a preferred angle exists it probably represents an average position, since some freedom of oscillation of the Fab arms about the hinge region has been shown to occur (6). The demonstration of a preferred conformation would help to answer the critical but as yet unresolved question of whether or not a definite conformational change occurs when an antibody binds to a multivalent antigen, and if so, in what direction might such a conformational change lie.

We report in this paper the results of our attempt to resolve the matter of a preferred conformation of IgG in solution. We have approached the problem by using singlet–singlet energy transfer to measure the distance between the two hapten binding sites (7). This method has been shown to be both theoretically and experimentally applicable to the measurement of molecular dimensions and has been successfully used in investigation of the properties of several macromolecules (7–10).

We have prepared a hybrid antibody molecule (11–13) in which one active site specifically bound ε-dimethylaminonaphthalenesulfonyl (DNS)-lysine, the energy donor, and the other site bound fluorescein, the energy acceptor. Fig. 1 shows a schematic representation of this molecule. The spectroscopic properties of these antibody-bound haptens are particularly well suited for the observation of long-range resonance-energy transfer between them. The quantum yield of DNS-lysine is considerably enhanced, while that of fluorescein is substantially reduced, resulting in a separation of lifetimes of greater than a factor of ten. Fig. 2 demonstrates that there is highly favorable overlap of the donor emission and acceptor absorption, which, together with the high quantum yield of the donor, makes energy transfer probable over large distances, i.e., in the order of 5–8 nm (50–80 Å).

MATERIALS AND METHODS

High affinity antifluorescein and anti-DNS antibodies (15) were elicited by repeated immunizations in the hind toepads of rabbits with fluorescein-hemocyanin (fluorescein isothiocyanate, Isomer I, Sylvana Co, Milburn, N. J., and keyhole limpet hemocyanin, Sigma) or DNS–hemocyanin (DNS-Cl, Pierce Chem. Co.) in complete Freund's adjuvant. Antibodies were purified by the following procedure from sera obtained more than 4 months after primary immunization. Experiments were performed at 4° unless otherwise noted.

Immunoglobulins were precipitated with ammonium sulfate (30% w/v) and dialyzed against a solution of phos-

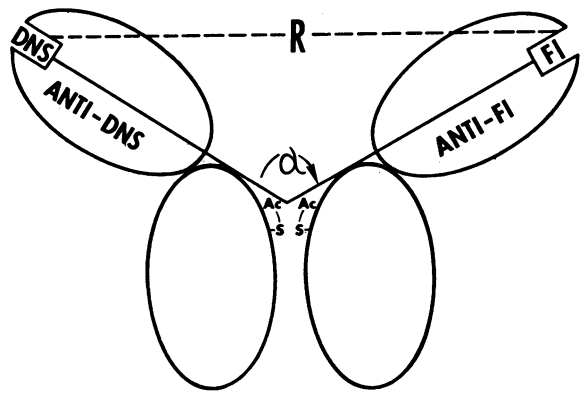

FIG. 1. Schematic representation of the structure of the anti-DNS–antifluorescein (anti-Fl) hybrid antibody–hapten complex. *R*, the distance between hapten-binding sites; α, the angle between the Fab moieties; *Ac*, carboxymethylene group.

Abbreviations: DNS, dimethylaminonaphthalenesulfonyl; BSA, bovine serum albumin; HSA, human serum albumin; BrAc, bromoacetyl.

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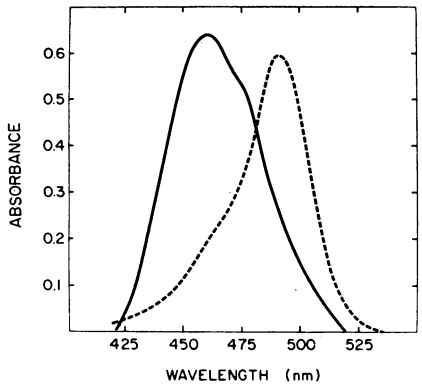

FIG. 2. Absorption spectrum (- - -) of 6.5 μM fluorescein bound to antifluorescein antibody in 0.01 M potassium phosphate–0.15 M NaCl buffer (pH 7.5). Emission spectrum (——) of 1.25 μM DNS-lysine bound to anti-DNS antibody in 0.01 M potassium phosphate–0.15 M NaCl buffer (pH 7.5).

phate buffered saline (PBS, pH 7.5, 0.01 M sodium phosphate and 0.15 M NaCl). Antibodies were selectively adsorbed by either a fluoresceinthiocarbamyl–bovine serum albumin (BSA)–bromoacetyl (BrAc) cellulose or naphthalenesulfonyl–human serum albumin (HSA)–BrAc cellulose immunoadsorbent (27), eluted with a solution of 1 M acetic acid for 45 min at room temperature, and extensively dialyzed against a solution of PBS. Upon binding with DNS-lysine, the resulting anti-DNS antibodies exhibited association constants greater than $2 \times 10^8\ M^{-1}$, as measured by the fluorescence enhancement method (15). The quantum yield of fluorescein bound to antifluorescein is about 5% that of free fluorescein, and upon binding, the emission wavelength of fluorescein is (red) shifted from 510 to 522 nm. Thus, by use of excitation and emission wavelengths of 485 and 510 nm, respectively, the fluorescence of free fluorescein can be preferentially detected. Association constants of antifluorescein and antibody fluorescein were found to be greater than $2 \times 10^8\ M^{-1}$.

Hybrid antifluorescein–anti-DNS antibody molecules were prepared by the method of Hong and Nisonoff (14) as described below. A solution of 76 mg of antifluorescein and 31 mg of anti-DNS antibodies, dissolved in 0.2 M Tris·HCl (pH 8.2) was reduced at room temperature for 75 min with 2-mercaptoethanol (final concentration, 0.01 M) and alkylated at room temperature for 1 hr with sodium iodoacetate (final concentration, 0.1 M.) The solution was dialyzed twice against 8 liters of 25 mM NaCl solution. 1 N HCl was added to bring the pH to 2.4; the solution was then stirred at room temperature for 15 min in order to separate the half molecules. Recombination of the half molecules (16) took place upon raising the pH of the solution by dialyzing it twice against 8 liters of 0.16 M borate–saline buffer (pH 8.0) and then twice against 8 liters of PBS. In a completely analogous fashion, a sample of reduced, alkylated, separated, and recombined anti-DNS antibody was prepared. Based on the protein concentration as determined from absorbance measurements, 73% of the theoretical number of binding sites of the recombined anti-DNS antibody bound DNS-lysine in a fluorescence-enhancement titration. This value is about the same as that found for native, purified anti-DNS antibody (74%).

A statistical treatment based upon the amounts of antifluorescein and anti-DNS antibodies used above predicted that the recombined mixture should contain 41% anti-DNS–antifluorescein hybrid, 8% recombined anti-DNS, and 50% recombined antifluorescein antibodies. Partial purification of the hybrid molecules from this mixture was achieved in the following manner. 45 mg of the antibody mixture was adsorbed on fluoresceinthiocarbamyl–BSA–BrAc cellulose immunoadsorbent. After equilibration at room temperature, 7 mg of anti-DNS antibody remained in the supernatant. After the immunoadsorbent–protein complex was washed repeatedly with PBS, the hybrid and antifluorescein antibodies were removed by elution at room temperature for 75 min with 0.2 M fluorescein dissolved in PBS. After exhaustive dialysis of the eluted solution against PBS for removal of the unbound fluorescein, 17.2 mg of protein were recovered, which corresponds to a protein concentration of 58 μM.

DNS-lysine was added to the anti-DNS antibody sites by fluorescence-enhancement titration of 5.3 ml of an 8.7 μM solution of the antibodies with 54 μl of a 278 μM DNS-lysine solution. The end point corresponded to an anti-DNS antibody binding site concentration of 14 μM. This value is 13% of the total number of binding sites that could be filled by both DNS-lysine and fluorescein. The shape of this titration curve did not significantly differ from that obtained when unreduced anti-DNS antibodies were titrated. This observation indicated that no major change in binding characteristics had occurred as a result of the hybridization procedure. Absorption measurements on the hybrid and antifluorescein antibody mixture showed that the concentration of fluorescein-containing sites was 68 μM, which corresponded to 67% of the total number of active sites for both haptens. Removal of the fluorescein-antifluorescein molecules was not deemed necessary (see *Results*).

Lifetime Measurements. Lifetime measurements were made by the monophoton technique (17). Excitation light was provided by an air gap relaxation oscillator lamp. The exciting light was unfiltered; emission was monitored at 450 nm with a Jarrel–Ash 0.25-m monochromater. For each sample, photon counts were accumulated until 60,000 counts were received in the peak channel. The ratio of stop to start pulses received by the time to amplitude converter was 0.05.

Analysis of the Data. We used the method of moments (18) to analyze the decay curves obtained. A computer program was used in conjunction with an IBM 360 computer. This is a batch-loading program similar to that of Isenberg and Dyson (18), with additional corrections of light scattering by the sample (Langlois, R., and Cantor, C., manuscript in preparation). The best fit of the data was obtained with the assumption of a single component decay.

Spectral Methods. Absorption spectra were obtained on a Cary model 11 recording spectrophotometer. Corrected emission spectra were obtained on a model 210 "Spectro" absolute spectrofluorometer (G. K. Turner Associates).

Fluorescence Titrations were performed on an Aminco–Bowman spectrophotofluorometer, equipped with an ellipsoidal condensing mirror, with solutions containing antibody concentrations of about 2 μM and a stock DNS-lysine concentration of 278 μM.

Precipitin Tests were performed by a modification (19) of the standard procedure (20) with the heterologus antigens fluorescein–BSA or DNS–bovine serum albumin.

Ultracentrifugation was performed at room temperature, in a Spinco model E analytical ultracentrifuge equipped with Schlieren optics. The protein concentration of all samples was 3 mg/ml.

Gel Electrophoresis in polyacrylamide gels was performed by the method of Weber and Osborn (21). The gel-pore size was selected to allow entry of molecules that have a molecular weight of less than 250,000. The buffer solution used in the reservoirs was 0.01 M sodium phosphate (pH 7.0) containing 1% sodium dodecyl sulfate. Current was applied for 2 hr at 8 mA per gel. Protein bands were developed with ethanolic solutions of Buffalo Black dye.

RESULTS AND CALCULATIONS

Hybrid preparation

The ideal situation for the purpose of measuring energy transfer in the hybrid molecule would be to investigate a solution containing only the hybrid antibody molecules. Purification of the high affinity antibodies used in these experiments, however, presented certain difficulties necessitating a compromise. The usual method for removal of an antibody bound to an immunoadsorbent is either by elution with an acid or by elution with a hapten that effectively competes for the occupied active sites. With reduced and alkylated antibodies, acid elution results in separation and rescrambling of the noncovalently bound halves of the hybrid molecules, and cannot be used. Hapten elution was therefore used. In theory, the mixture of hybrid and recombined antifluorescein and anti-DNS antibodies could have been completely separated from one another by absorption first onto a fluoresceinthiocarbamyl–BSA–BrAc cellulose immunoadsorbent, elution with fluorescein, absorption onto naphthalenesulfonyl–HSA–BrAc cellulose immunoadsorbent, and elution with DNS-lysine. However, this procedure would have resulted in a hybrid preparation that would have had all or most of the sites filled with hapten. Since DNS-lysine has a relatively low extinction coefficient at 340 nm (4.57×10^3, ref. 24) and since fluorescein also has significant absorption at this wavelength (22), it would have been difficult in the hybrid to determine accurately the concentration of anti-DNS sites. We therefore chose to remove only the recombined anti-DNS antibody from the mixture, since the presence of this species would invalidate the energy transfer measurements. Absorption onto fluoresceinthiocarbamyl–BSA–BrAc cellulose immunoadsorbent and elution with fluorescein left the anti-DNS antibody sites of the hybrid free and available for titration. The presence of recombined antifluorescein antibody does not interfere with the measurements since the quantum yield of bound fluorescein emission is extremely low (about 5% of that of free fluorescein). Furthermore, since the lifetime of emission of bound fluorescein is very short, in the order of 2–3 nsec, fluorescein does not affect measurements of the relatively long DNS decay.

Separation of antibody into half molecules at low pH, recombination into whole molecules at neutral pH, and the extent of possible aggregate formation upon recombination was followed by two treatments: analytical ultracentrifugation and gel electrophoresis. It was important to demonstrate that little or no aggregation had occurred in the hybrid preparation. If any appreciable aggregation did occur, either between hybrid molecules themselves or between hybrid and antifluorescein antibody molecules, there would have been a possibility that a DNS-containing active site would have been relatively close to a fluorescein-containing active site. Such a situation would have resulted in an extraneous lowering of the DNS lifetime, and might have masked any small reduction in lifetime due to transfer between sites in the hybrid. The sedimentation pattern of a reduced and alkylated mixture of anti-DNS and antifluorescein antibody in 25 mM NaCl at pH 2.4 showed a single symmetric peak with a sedimentation coefficient $s_{20,w} = 3.4$; thus, at least 90% of the material at this pH consisted of only half molecules. As a check, comparative sodium dodecyl sulfate gel electrophoresis was performed with the same sample and native, unreduced antifluorescein antibody. Two bands were observed in the reduced and alkylated sample; the major band (about 90–95% of the total material) consisted of half molecules and traveled about twice the distance traveled by the unreduced antibody, whereas a minor band (5–10% of total material) consisted presumably of dissociated chains and traveled ahead of the major band. After recombination of this same sample, the sedimentation pattern showed a single band with a value of $s_{20,w} = 6.7$, a tail on the slower sedimenting side, and no faster sedimenting material. These results showed that whole molecules had reformed after the pH was raised and that the extent of aggregation in this sample could not have been more than 5%. Any remaining nonrecombined subunits of anti-DNS antibody molecules were removed from the hybrid preparation by adsorption to fluoresceinthiocarbamyl–BSA–BrAc cellulose.

The methods that we used to show hybrid formation are based on the principle that the hybrid, since it contains one site specific for DNS and one for fluorescein, should, after hapten elution from the fluoresceinthiocarbamyl–BSA–BrAc cellulose immunoadsorbent, still have a free DNS site, but should not be precipitable with DNS–BSA. Fluorescence enhancement titration with DNS-lysine of the hybrid and antifluorescein antibody mixture showed that 13% of the total sites bound DNS-lysine. Since 67% of the total sites were filled with fluorescein, 80% of the total number of sites could be accounted for. The remaining 20% presumably were either denatured at some stage during the preparative procedure, or were present as subunits of antifluorescein antibody. (Any subunits of anti-DNS antibody were removed by adsorption to fluoresceinthiocarbamyl–BSA–BrAc cellulose.)

A prediction of the concentration of titratable DNS-binding sites, based on the assumption that recombination of half molecules was random and on the observation that 20% of the total number of sites was inactivated, gave a concentration of 19 μM. Since the observed concentration was 14 μM, these figures are in reasonably good agreement.

A precipitin test of the hybrid and antifluorescein antibody solution with DNS–BSA was negative, which showed that the DNS sites were not present in the form of recombined anti-DNS antibody, which was found instead in the supernatant of the original hybrid, antifluorescein, and anti-DNS mixture, after addition of fluoresceinthiocarbamyl–BSA–BrAc cellulose immunoadsorbent.

Calculation of R_0

For the determination of the extent of energy transfer between DNS-lysine and fluorescein in their respective active sites in the hybrid, it was necessary to know the theoretical separation

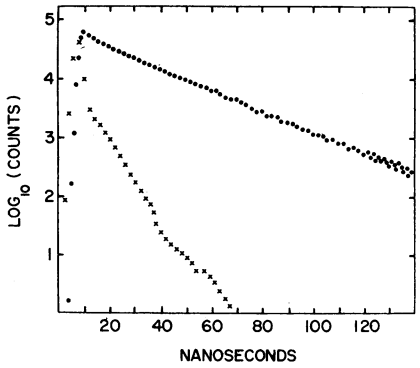

FIG. 3. Nondeconvoluted fluorescence decay of reduced and alkylated, separated and recombined anti-DNS antibody–DNS-lys complex (· · ·). The concentration of antibody was 0.4 mg/ml; that of bound DNS-lysine, 3.9 μM. Exciting light was polychromic; emission was monitored at 450 nm. Time base: 1 nsec per channel. The data points corresponding to approximately every second channel are shown. Flash-lamp decay (+ + +).

distance, R_0, at which 50% of the excitation energy of the donor is transferred to acceptor. This value was obtained from the expression (23):

$$R_0 = (8.77 \times 10^{-25} K^2 \phi_D J / n^4)^{1/6} \quad (1)$$

J, the overlap integral of the donor emission and acceptor absorption, was calculated by comparison of the weight of graph paper under the overlap of a corrected and normalized emission spectrum of anti-DNS antibody–DNS-lysine and the absorption spectrum of antifluorescein antibody–fluorescein to the weight of paper under a known area of proper units. The value of J thus obtained was 1.81×10^{-10} M^{-1} cm^6. The refractive index, n, of the solvent was assumed to be 1.5 (25). The value of K^2, the orientation factor, was assumed to be equal to $^2/_3$; the validity of this assumption is presented in the *Discussion*. The quantum yield, ϕ_D, of bound DNS-lysine in the absence of energy transfer is variable (15) due to the heterogeneity of anti-DNS antibodies. For high affinity rabbit anti-DNS antibody, however, an average value for the quantum yield of the donor can be obtained. Our data and those of Parker *et al.* (15) give $\phi_D = 0.60 \pm 0.1$. With these values, R_0 was calculated to be 4.8 ± 0.2 nm (48 ± 2 Å). This is one of the highest reported values of R_0 for an experimentally feasible system (26).

Energy transfer

The extent of energy transfer between DNS and fluorescein was determined from a comparison of the fluorescence lifetime of DNS in recombined anti-DNS antibody, which contained no acceptor, and in the hybrid. To see whether energy transfer might occur between donor and acceptor in separate antibody molecules, the lifetime of DNS in a *mixture* of anti-DNS and antifluorescein antibody molecules was also measured. Fig. 3 shows a typical *nondeconvoluted* fluorescence decay curve of a solution of recombined anti-DNS antibody molecules. The values of the mean lifetimes obtained for these solutions were 23.9, 23.6, and 23.1 nsec, respectively. Within the error of the method (3%, ref. 17), these values are identical, indicating that no energy transfer between chromophores had occurred. These values are in excellent agreement with the decay time of 23.1 nsec obtained by Yguerabide *et al.* (6) for DNS-lysine in heterogeneous active sites of rabbit anti-DNS IgG.

DISCUSSION

The lack of observable energy transfer between the two chromophores (fluorescein and DNS) does not allow an exact measurement of the separation distance, R, between them. It does, however, allow a lower limit of the separation distance, R_m, to be set, and makes it possible to choose between several models of IgG conformation.

The value of R_m will be a function of the lower limit of detection of energy transfer and this in turn will be dependent on both the value of R_0 of the donor–acceptor pair and the sensitivity of the method of lifetime measurement. The separation distance between chromophores, R, as a function of the rate of energy transfer can be given by:

$$R = R_0/(k\tau)^{1/6} \quad (2)$$

where R_0 is the critical transfer distance obtained from theory, τ is the donor lifetime in the absence of energy transfer, and k is the first order rate constant of energy transfer given by:

$$k = 1/\tau' - 1/\tau \quad (3)$$

where τ' is the lifetime of the donor in the presence of energy transfer. The uncertainty of a lifetime measurement by the monophoton technique is about 3% (17). Thus, the smallest reduction in the lifetime of DNS that could be accurately measured is 0.8 nsec. To be conservative, we have assumed this value to be 1 nsec. If we assume that the minimum measurable decrease in the donor lifetime is 1 nsec, we obtain the relationship:

$$R_m = 1.7\, R_0 \quad (4)$$

The value of R_0 depends on the value of the orientation factor K^2 that may vary from 0 to 4 depending on the orientation of the transition dipole moments of the chromophores. When these moments are randomly oriented, $K^2 = ^2/_3$. The actual value of K^2 for the hybrid antibody preparation is undetermined experimentally, since the directions of the transition moments in fluorescein and DNS and the orientation of the active sites in the Fab moieties are unknown. However, $K^2 = ^2/_3$ is probably a good approximation. Ygueribide *et al.* (6) have shown that anti-DNS antibodies show segmental flexibility with each Fab fragment traversing, in times of nanoseconds, an angular range of about 30°. Such flexibility will lead to some randomization of the transition moment dipoles during the relatively long lifetime of the donor's excited state. Also, due to the known heterogeneity of both anti-DNS and antifluorescein antibody populations (15, 28), the orientation of the active site in the Fab moiety for a given specificity is probably not the same in all of the molecules. Even if the orientations of dipoles were not completely randomized, however, $K^2 = ^2/_3$ is still probably a good approximation. We have found from calculations of K^2 as a function of several different possible dipole orientations and varying angles between Fab fragments that most of the values of K^2 were about 1.0. Since R_0 is a function of the sixth root of K^2, a value of $K^2 = 1$ would also lead to approximately the same value of R_0. Until the orientation factor is evaluated experimentally, however, the assumption that $K^2 = ^2/_3$ must be viewed with due caution.

From Eq. 4, R_m was found to be 8.2 ± 0.3 nm (82 ± 3 Å). The actual minimum distance is probably greater since the Fab fragments exhibit segmental flexibility. The proportion of this segmental motion that is directly along the line drawn between the active sites is unknown. However, we can make the reasonable assumption that half of the 30° angular range is in this direction. Then, if the average position were at R = 8.2 nm (82 Å), some energy transfer would be observed, since there would be a high probability that the donor–acceptor pair would spend some time at a shorter distance. If one assumes that the length of the Fab fragment is 7.0 nm (70 Å, refs. 1, 29), a simple trigonometric analysis adds an additional 1 nm (10 Å) to the minimum separation distance, and R_m = 9.2 nm (92 Å). If one further assumes that the active site lies exactly on the tip of this fragment (1, 2, 5), a minimum angle, α_M, between the Fab fragments can be set. If R_m = 9.0 nm (90 Å), then α_M = 80°. If all of the segmental motion is directly along the line of centers, then R_m = 10.2 nm (102 Å).

From similar considerations, it is unlikely that any significant proportion of molecules exist in conformations in which α_M is less than 80°. We can therefore conclude that if such a conformation occurs in antibody combined with antigen (2), it is due to a definite conformational change.

Our results also demonstrate that the active sites cannot lie more than 2.5 nm (25 Å) [and more likely not more than 2.0 nm (20 Å)] from the tips of the Fab fragments. If they did, even at the greatest separation distance that would occur with α = 180°, energy transfer between sites would then be observed.

It is instructive to compare these results with the separation distances and angles obtained by other investigators using other techniques. Valentine and Green (2) in their electron microscopic investigation of rabbit anti-DNP antibodies bound to divalent hapten proposed a *Y*-shaped conformation in which the length of the Fab fragment was 6 nm (60 Å) and α varied from 10 to 180°, depending on the polymeric size of the complex. More recently, Green (1) has suggested that the length of the Fab fragment is 7 nm (70 Å) and that α in *free antibody* molecules is less than 60°. The recent results of Yguerabide *et al.* (6) on nanosecond emission anisotropy support the concept of a flexible molecule with each Fab traversing an angular range of about 30° in nanoseconds. The transient electric birefringence results of Cathou and O'Konski suggested that, in solution, α may lie between 130 and 180°, and that the length of the Fab fragment is of the order of 6 nm (60 Å) (3). The model proposed by Pilz *et al.* (4), based on low-angle x-ray scattering data of a human IgG_1 myeloma protein in solution is a *T*-shaped molecule with α invarient at 180°. In their model the length of the Fab fragment is 9.8 nm (98 Å), leading to a separation distance of about 20 nm (200 Å). These dimensions are significantly larger than those suggested for rabbit IgG. The 0.6-nm (6-Å) resolution x-ray crystallographic study of Samra *et al.* (29) of another human myeloma IgG_1 are consistent with a *T*-shaped structure in the *crystal*, and a distance between sites of about 14 nm (140 Å) and α = 180°.

The results reported here agree that an open *Y*- or *T*-shaped IgG molecule occurs in solution, that the distance between active sites is 9 nm (90 Å) or greater, and that the angle between Fab fragments is at least 80°. The only proposed model that is in disagreement with these results is that suggested by electron microscopy. Hence, we must conclude that the conditions used for production of the electron microscopy model distort the molecule that is not bound by a hapten either by complexing the molecule with multivalent hapten and/or by drying it on a grid.

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