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Fluorescence Polarization of Human γG-Immunoglobulins*

Joel K. Weltman† and Gerald M. Edelman

ABSTRACT: The fluorescence polarization of 1-dimethylaminonaphthalene-5-sulfonyl conjugates of human γG-immunoglobulins (DNS-IgG) has been measured under various conditions. Rotational relaxation times at 25° were calculated from experiments in which the viscosity of the solvent was varied by altering the temperature. Different rotational relaxation times were obtained when the solvent viscosity was altered by addition of sucrose at fixed temperatures. The results are explained by assuming that DNS groups covalently bound to the immunoglobulin undergo thermally activated rotations independent of the rotation of the macromolecule. This hypothesis was tested by experiments which showed that addition of a DNS conjugate of ε-aminocaproic acid to solutions of DNS conjugates of immunoglobulin mimics the effect of temperature on the immunoglobulin conjugates in the absence of any fluorescent small molecules. Moreover, on the basis of this hypothesis, the polarizations in heated solutions were calculated from polarizations of DNS-IgG measured in sucrose solutions at fixed temperatures (sucrose isotherms). A rotational relaxation time of approximately 221 nsec was obtained both for human γG-immunoglobulin and human γG-myeloma protein. This value is in agreement with reported estimates obtained by other relaxation methods and is considerably higher than values previously obtained from fluorescence polarization measurements. An effect of the wavelength of excitation on the polarization of fluorescence was also noted. This effect appeared to be related to the presence of different environments of covalently bound DNS groups. The present findings are consistent with rodlike models of the IgG and incompatible with extreme molecular flexibility.

The fluorescence polarization of 1-dimethylaminonaphthalene-5-sulfonyl conjugates[1] of γG-immunoglobulins has been studied in a number of laboratories (Steiner and Edelhoch, 1962; Chowdhury and Johnson, 1963; Winkler, 1965). The results of such studies have been used to compute a rotational relaxation time (ρ_h) of the macromolecule. In general, the reported values of ρ_h (approximately 100 nsec) are lower than would be expected if the γG-immunoglobulin molecule behaved as a rod-shaped particle (Edelman and Gally, 1964) with dimensions of 240 × 57 × 19 A (Kratky *et al.*, 1955). This discrepancy has usually been interpreted as an indication that the DNS group is attached to macromolecular regions that are capable of independent rotation. On these and other grounds, it has been proposed that the γG-immunoglobulin molecule is a flexible structure (Noelken *et al.*, 1965).

An alternative explanation for the observed values of ρ_h is that covalently bound DNS groups undergo thermally activated rotations independent of macromolecular rotations (Weber and Teale, 1965). In the present study, we provide evidence that thermally activated rotation of conjugated DNS groups is largely responsible for lowering the ρ_h values of γG-immunoglobulins. Using a theory which relates fluorescence polarization data obtained at different temperatures and viscosities (Weber, 1952a), the ρ_h of immunoglobulin is estimated to be approximately 221 nsec. This value is consistent with the dimensions and shape parameters calculated from data obtained by a variety of methods (Kratky *et al.*, 1955; Noelken *et al.*, 1965). Although γG-immunoglobulin molecules may have some degree of flexibility, the fluorescence polarization of DNS conjugates does not provide compelling evidence that it exists.

Materials and Methods

All chemicals were reagent grade unless otherwise

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[1] Abbreviations used: DNS, 1-dimethylaminonaphthalene-5-sulfonyl; ρ_h, rotational relaxation time. Unless otherwise indicated, ρ_h was calculated for a temperature of 25°. EACA, ε-aminocaproic acid; IgG, γG-immunoglobulin; Eu, γG-myeloma protein.

specified. Acetone, ethanol, benzene, and *p*-dioxane were redistilled on a 120-cm fractionating column. Their purity was assessed by checking their refractive indices. ε-Aminocaproic acid (Mann, lot no. 1934) was recrystallized from aqueous solutions at room temperature by adding sufficient ethanol to initiate crystallization. The crystals were washed with ethanol and dried *in vacuo* over NaOH.

Protein and DNS Concentrations. The concentrations of γG-immunoglobulin and of DNS groups were estimated from their respective absorbancies at 280 and 325 mμ. An absorbancy of 13.6 (Crumpton and Wilkinson, 1963) for a 1% solution of γG-immunoglobulin in a 1-cm light path was used in all calculations. An extinction coefficient of 4.3×10^6 cm^2 (g-mole)$^{-1}$ was used for the DNS group (Weber, 1952b). In calculating the concentration of DNS groups, correction was made for protein absorbancy at 325 mμ. Unless otherwise indicated, a molecular weight of 151,000 was assumed for γG-immunoglobulin (Pain, 1963).

Electrophoresis. Analytical electrophoresis on cellulose acetate was performed for 1.5 hr at 20° in barbital buffer having an ionic strength of 0.05 M at pH 8.6, under a potential gradient of 10 v/cm. Cellulose acetate strips were examined for fluorescence under a Woods lamp and stained for protein with 0.2% Ponceau S in 3% trichloracetic acid. Two of the DNS conjugates of γG-immunoglobulin and myeloma protein were purified by zone electrophoresis on polyvinyl chloride (Kunkel, 1954).

Determination of Sedimentation Coefficients. Sedimentation coefficients were estimated from the sedimentation velocity in a sucrose density gradient by the method of Martin and Ames (1961) unless otherwise specified. *Escherichia coli* alkaline phosphatase was used as a marker (Olins and Edelman, 1964). In the case of two of the DNS conjugates (IgG-DNS-2 and Eu-DNS-1) the sedimentation coefficient ($s_{20,w}$) was calculated from sedimentation velocity experiments on 0.5% solutions in 0.05 M Tris–0.15 M NaCl (pH 8.0). The measurements were obtained with a Model E Beckman-Spinco analytical ultracentrifuge equipped with temperature control and schlieren optics and operated at 52,000 rpm.

γG-Immunoglobulin. γG-Immunoglobulin (IgG) was Cohn fraction II (Lederle Laboratories, Pearl River, N. Y., lot C-863). A single component was detected by electrophoresis on cellulose acetate. In addition to the major 7S component, this sample contained approximately 10% of material with a sedimentation coefficient of 9 S. γG-Myeloma protein (Eu) was isolated from the serum of a patient with multiple myeloma by adding $(NH_4)_2SO_4$ to 33% saturation. Further steps in the purification and characterization of IgG and Eu are described below.

Preparation and Characterization of DNS-Cl. DNS-Cl was prepared by the method of Weber (1952b) as modified by Laurence (1957). The melting point was 67° (capillary tube, uncorrected). The DNS-Cl was dissolved in acetone and centrifuged before use to remove insoluble hydrolysis products. This sample of DNS-Cl was used to prepare the DNS conjugate of γG-immunoglobulin denoted IgG-DNS-1. All other conjugates were prepared from DNS-Cl (K & K Laboratories, Plainview, N. Y., lot no. 1290) which was purified by means of thin layer chromatography on Eastman Chromagram sheets (type K301R, Eastman Organic Chemicals Co., Rochester, N. Y.) using benzene as the solvent. The R_F of DNS-Cl in this system was 0.83. DNS-Cl was eluted from the chromatogram with benzene which was then evaporated in a stream of prepurified N_2. The resulting crystals were redissolved in *p*-dioxane.

Preparation and Characterization of DNS Conjugates of γG-Immunoglobulins. DNS-Cl dissolved in acetone or *p*-dioxane was added to a 0.5–1.0% solution of γG-immunoglobulin in 0.1 M $NaHCO_3$ at 4°. The ratio of DNS-Cl to protein was 1:100 by weight and the final concentration of acetone or dioxane was less than 1%. Conjugation of DNS groups to the protein was allowed to proceed for 1–2 hr at 4° with gentle stirring.

The conjugated proteins were separated from hydrolyzed and unreacted DNS-Cl by dialysis against 0.05 M Tris and 0.15 M NaCl (pH 8.0, Tris-NaCl buffer). In one case (Eu-DNS-2) gel filtration on a 2.5 × 35 cm column of Sephadex G-25 (Pharmacia Chemicals, Inc., Uppsala, Sweden) in Tris-NaCl buffer was used instead of dialysis. Each DNS conjugate of γG-immunoglobulin was examined by electrophoresis on cellulose acetate and was free of detectable contaminants.

The DNS conjugate of γG-immunoglobulin, designated IgG-DNS-1, was prepared directly from the commercial Cohn fraction II. The molar ratio of DNS to protein in the conjugate was 2.4. Examination of IgG-DNS-1 by ultracentrifugation in density gradients showed that the conjugated product was free of aggregated material. The fluorescence polarization of solutions of IgG-DNS-1 was independent of concentration between 6.6×10^{-5} and 3.3×10^{-6} M (1.0–0.05%).

IgG-DNS-2 and Eu-DNS-1 were the respective conjugates of aliquots of IgG and of Eu which were prepared by electrophoresis on polyvinyl chloride prior to reaction with DNS-Cl. The value of $s_{20,w}$ determined on 0.5% solutions by means of analytical ultracentrifugation was 7.2 S for IgG-DNS-2 and 6.9 S for Eu-DNS-1. There was no evidence of aggregates. The molar ratio of DNS groups to protein was 2.8 for IgG-DNS-2 and 3.1 for Eu-DNS-1.

A sample of Eu was also prepared by means of chromatography on DEAE-cellulose in 0.03 M phosphate buffer (pH 8.2) (Peterson and Sober, 1962). The resulting γG-immunoglobulin was used to prepare the conjugate denoted Eu-DNS-2. The molar ratio of DNS groups to protein in Eu-DNS-2 was 3.3. The solvents used for polarization of fluorescence measurements were either Tris-NaCl buffer or sucrose solutions made with Tris-NaCl buffer as described below.

Emission spectra on the derivatives of human γG-

immunoglobulins were obtained using the exciting wavelengths 325, 345, and 365 mμ. The shapes of these spectra were the same as those reported by Winkler (1965) for DNS conjugates of rabbit γG-immunoglobulin. No differences were observed between the spectra obtained in Tris-NaCl buffer and in Tris-NaCl buffer containing 41.5% sucrose.

DNS Conjugates of ε-Aminocaproic Acid. The DNS conjugate of ε-aminocaproic acid (DNS-EACA) was synthesized under the same conditions as the conjugates of γG-immunoglobulins. The following procedure was used to fractionate the DNS-EACA conjugate from EACA and the other reagents. HCl (1 N) was added to the solution in which conjugation was carried out until the pH was 1.3. This acidified solution was extracted with benzene. The benzene phase was removed and extracted with an aqueous solution of NaOH at pH 11.1. The separated aqueous phase was readjusted to pH 1.3 with 1 N HCl and the benzene extraction was repeated. The benzene phase of this second extract was removed and evaporated in a stream of prepurified N_2 and the resulting residue of DNS-EACA was dissolved in Tris-NaCl buffer. The preparation of DNS-EACA was subjected to thin layer chromatography in chloroform–benzyl alcohol–acetic acid (7:3:0.3, v/v). The chromatogram was examined for fluorescence under a Woods lamp and then stained with ninhydrin to detect EACA. A single fluorescent component (R_F 0.68) which was not contaminated by EACA was detected.

Determination of Sucrose Concentration and Calculation of Viscosity (η). In experiments in which the viscosity was varied by means of the addition of sucrose, the sucrose concentration of each solution was measured with an Abbé refractometer. The sucrose solutions were prepared using Tris-NaCl buffer as solvent.

The viscosity of Tris-NaCl buffer was taken to be that of water at the same temperature. The viscosity of each sucrose solution was calculated from the nomogram of Othmer and Silvis (1948).

Fluorescence Polarization and Determination of ρ_h. The fluorescence polarization of DNS conjugates of γG-immunoglobulin in Tris-NaCl was measured with a recording spectrofluorometer which has been described elsewhere (Rosen and Edelman, 1965). A 1600-w high-pressure xenon lamp was the light source. The entrance and exit slits of the excitation monochromator were fixed at 2 mm. The wavelength of the exciting light was varied from 325 to 385 mμ as described in the Results section.

Glan–Thompson prisms were used as polarizer and analyzer. In order to minimize irradiation by stray light, a Corning 7-54 filter was interposed between the excitation monochromator and the polarizer. To minimize the contribution of scattered exciting light a Corning 3-71 filter and a 2-mm layer of 1.0 M $NaNO_2$ were interposed between the analyzing prism and the detection photomultiplier. Samples were placed in a quartz cuvet within a temperature-controlled holder. Solutions were heated from 4 to 45° and the temperature was read to ±0.5° using a nylon-coated thermistor probe (Yellow Springs Instrument Co., Yellow Springs, Ohio) immersed in the sample.

The polarization (p) is

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \tag{1}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the emitted light with electric vectors parallel and perpendicular, respectively, to the electric vector of the incident light.

According to Perrin (1926)

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{RT\tau}{\eta V}\right) \tag{2}$$

where $1/p_0$ is the reciprocal of the fluorescence polarization in the absence of molecular rotation, R is the gas constant, T is the absolute temperature, τ is the lifetime of the first singlet excited state of the fluorescent group, η is the viscosity of the solution, and V is the molecular volume of the fluorescent molecule.

For a spherical macromolecule

$$\rho_h = \frac{3\eta}{RT}V \tag{3}$$

where ρ_h is the relaxation time of the rotating unit. Weber (1952a) has extended the theory of Perrin to the case of an ellipsoidal macromolecule, where ρ_h is the harmonic mean of the three rotational relaxation times corresponding to the axes of the ellipsoid.

Substitution of eq 3 into eq 2 yields

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right) \tag{4}$$

The value of the intercept on the ordinate of a plot of $1/p$ *vs.* T/η is $1/p_0$ for heated solutions. In the case of plots of $1/p$ *vs.* T/η at constant temperature (*i.e.*, isotherms in sucrose solutions) the intercept on the ordinate is called $1/p_0'$ which takes the place of $1/p_0$ (see Discussion). According to the above equations, ρ_h may be calculated from the plots of $1/p$ *vs.* T/η using a value of 13 nsec for the lifetime of the excited state of DNS groups at 25° (Steiner and Edelhoch, 1962). In the present paper ρ_h was calculated for T/η equal to 3.34×10^{-4} deg poise^{-1} which is the value for water at 25°.

Measurement of Fluorescence Intensity. In the experiments in which relative fluorescence intensity was measured as a function of temperature, a 250-w mercury–xenon lamp was used to excite the sample at 366 mμ with slits of the excitation monochromator set at 2 mm (dispersion = 3.3 mμ/mm at the exit slit). An analyzing monochromator with slits of 6 mm (dispersion, 1.6 mμ/mm) was used to isolate the fluorescence emitted at 500 mμ.

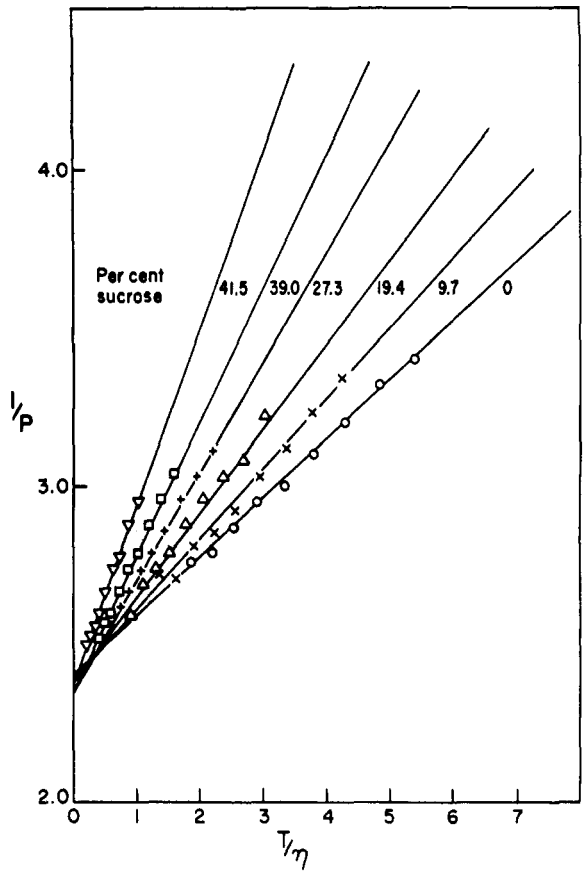

FIGURE 1: Reciprocal of the polarization of fluorescence ($1/p$) as a function of temperature divided by viscosity (T/η in deg poise^{-1} $\times$ 10^{-4}) for human DNS-γG-immunoglobulin in solutions containing various concentrations of sucrose. Preparation is IgG-DNS-1 at 3.2×10^{-5} M; wavelength of excitation 365 mμ. The curves were obtained by heating each solution in the range 5–45° (heating curve). The different symbols refer to experimental points obtained at different sucrose concentrations.

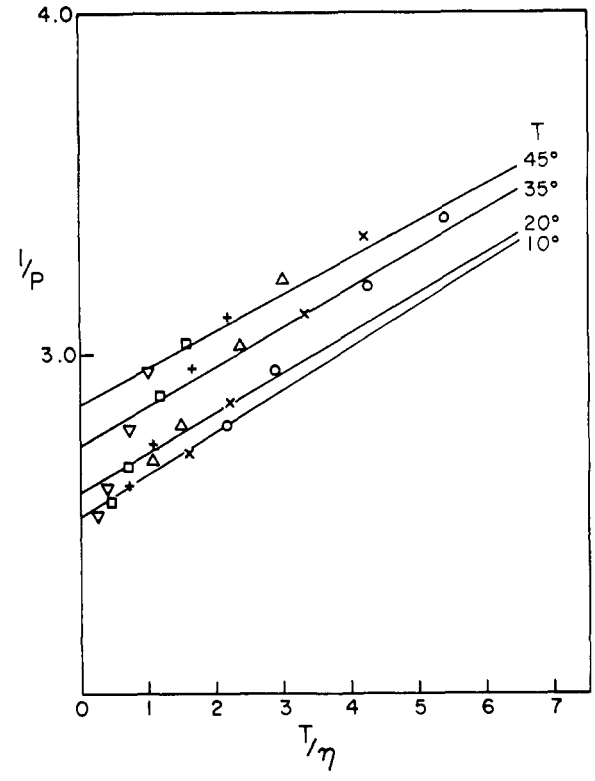

FIGURE 2: Isotherms of $1/p$ values obtained from different sucrose solutions of human γG-immunoglobulin. Data taken from same experiment described in Figure 1. Temperatures are indicated to the right of each isotherm. (○) no sucrose present. (×) 9.7% sucrose. (△) 19.4% sucrose. (+) 27.3% sucrose. (□) 39.0% sucrose. (▽) 41.5% sucrose.

Results

The effect of temperature on the polarization of fluorescence of γG-immunoglobulin conjugates is given in Figure 1. Solutions of IgG-DNS-1 were prepared in the presence of increasing amounts of sucrose as well as in the absence of sucrose. They were heated over the range from 5 to 45°. The slopes of the heating curves increased with increasing sucrose concentration although all curves could be extrapolated to approximately the same value of $1/p_0$. Inasmuch as the value of T/η at any constant temperature is different in the different sucrose solutions, a set of isotherms may also be plotted (Figure 2). In this case, the slopes are not as steep, vary less, and each isotherm extrapolates to a different value ($1/p_0'$) on the ordinate.

The data given in Figures 1 and 2 were obtained with an excitation wavelength of 365 mμ. Similar data were also obtained with excitation wavelengths of 325, 345, and 385 mμ. The values of $1/p_0$ and the calculated ρ_h values are given as a function of wavelength of excitation and sucrose concentration in Table I. Although at any particular wavelength the value of $1/p_0$ does not vary greatly with sucrose concentration, the value of $1/p_0$ decreases as the wavelength is increased. The slopes of the heating curves at any sucrose concentration were also found to decrease as the wavelength of excitation was increased. These variations affect the ρ_h values as shown in Table I. At all sucrose concentrations the calculated ρ_h increased with longer wavelengths of excitation. The data from the heating curves in Figure 1 and Table I may be summarized by stating that ρ_h decreases with increasing sucrose concentration and increases with increasing wavelengths of excitation.

It is evident that a single unequivocal value of ρ_h cannot be calculated for DNS conjugates of γG-immunoglobulin unless the effects of temperature, sucrose concentration, and exciting wavelength are analyzed. An effort was therefore made to isolate these variables. One possibility arises at the outset of such an analysis. γG-immunoglobulins are known

TABLE I: $1/p_0$ and ρ_h Calculated from Heated Solutions of γG-Immunoglobulin.[a]

% Sucrose	$1/p_0$				ρ_h (nsec)			
	λ_e 325	345	365	385	325	345	365	385
0.0	2.92	2.62	2.41	2.27	92	103	131	151
9.7	2.86	2.54	2.40	2.30	74	80	110	142
19.4	2.87	2.54	2.39	2.25	59	64	90	100
27.3	2.88	2.58	2.37	2.22	47	53	70	72
39.0	2.89	2.57	2.37	2.23	38	43	57	65
41.5	2.91	2.59	2.39	2.22	29	30	43	45

[a] ρ_h is calculated for $T/\eta = 3.34 \times 10^{-4}$ deg poise^{-1} which is the value for water at 25°. λ_e, wavelength of excitation in millimicrons. Preparation is IgG-DNS-1 at 3.2×10^{-5} M (see Materials and Methods).

(Kabat, 1961; Edelman and Kabat, 1964) to be extremely heterogeneous. It is possible that the effects of sucrose and exciting wavelength might reflect the conjugation of DNS groups to different portions of different immunoglobulins in the heterogeneous preparation. A homogeneous γG-immunoglobulin from a patient with multiple myeloma was, therefore, investigated in the same way as the normal γG-immunoglobulin. As shown in Table II, $1/p_0$ and ρ_h varied in the same way for the myeloma protein and the heterogeneous normal protein. A decrease in the value of ρ_h with increasing sucrose concentration was also observed for the myeloma protein. One may conclude that wavelength effects and the sucrose effects are not consequences of the chemical heterogeneity of normal γG-immunoglobulin.

TABLE II: $1/p_0$ and ρ_h Calculated from Heated Solutions of γG-Immunoglobulin (IgG) and Myeloma Protein (Eu).[a]

λ_e	$1/p_0$		ρ_h (nsec)	
	IgG	Eu	IgG	Eu
325	2.95	2.80	63	80
335	2.93	2.72	70	81
345	2.65	2.61	73	87
355	2.54	2.57	82	101
365	2.46	2.54	93	116
375	2.44	2.47	105	117
385	2.39	2.45	113	125

[a] ρ_h is calculated for $T/\eta = 3.34 \times 10^{-4}$ deg poise^{-1} which is the value for water at 25°. λ_e, wavelength of excitation in millimicrons. Preparations are IgG-DNS-2 at 6.2×10^{-5} M and Eu-DNS-1 at 6.9×10^{-5} M in Tris-NaCl buffer (see Materials and Methods).

A more likely hypothesis (Wahl, 1962; Gottlieb and Wahl, 1963) is that some of the covalently attached DNS groups are free to rotate, *i.e.*, are not rigidly bound to the γG-immunoglobulin. This would imply that the heating curves reflect some average effect of the rotation of the macromolecule and the thermally activated rotation of certain DNS groups. In that case, for a particular value of T/η, the relative contribution of freely rotating DNS groups to the polarization would increase with increasing temperature. Qualitatively, this would explain the decrease in apparent ρ_h as the viscosity was increased with sucrose.

The presence of freely rotating DNS groups on γG-immunoglobulin is difficult to verify directly. One might expect, however, to obtain the same general effects on the heating curves by addition of DNS conjugates of some small molecule to solutions of the protein conjugate. Accordingly, experiments were performed in which increasing amounts of DNS-EACA were added to solutions of Eu-DNS-2 (Figures 3 and 4). As predicted from theoretical considerations (Weber, 1952a) the slopes of the heating curves (Figure 3) are increased by the addition of increasing amounts of free fluorescent small molecules, without changing the value of $1/p_0$. Of particular relevance is the finding that the sucrose isotherm at 25° is shifted upward in more or less parallel fashion as DNS-EACA is added to DNS conjugates of myeloma protein (Figure 4). The effect on the isotherm produced by added DNS-EACA mimics that obtained with DNS-protein alone at different temperatures (Figure 2).

The presence of nonrigidly bound DNS groups on the protein surface might be expected to result in increased quenching of fluorescence as the temperature is raised. This effect was observed for Eu-DNS-2 (Figure 5), as well as for conjugates of the γG-immunoglobulin. The intensity of fluorescence decreased considerably over the temperature range 5–45°; this temperature dependence was the same for both the normal and the myeloma protein. Over the same temperature range there was no change in the shape of the emission spectrum or in the absorbancy at 366 mμ. Thus, the quenching of fluorescence as indicated

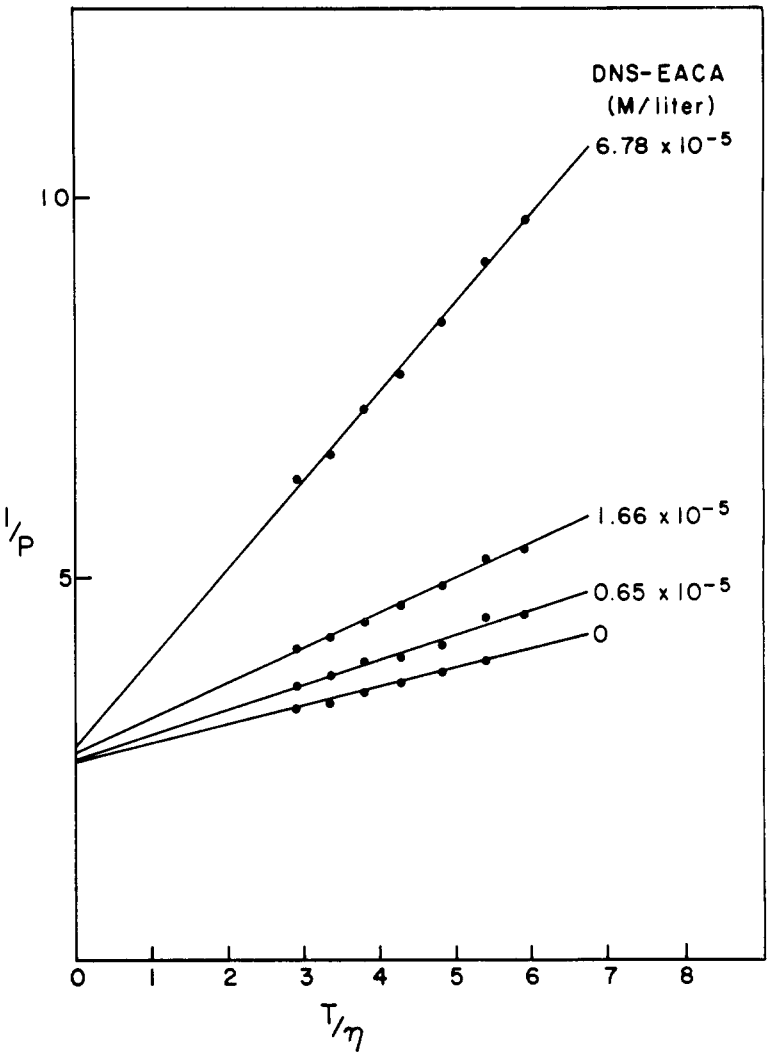

FIGURE 3: Heating curves depicting change in polarization after addition of DNS conjugate of ε-aminocaproic acid (DNS-EACA) to a DNS conjugate of human γG-myeloma protein (Eu-DNS-2, 2.8 × 10^{-5} M) in Tris-NaCl buffer. The concentration of DNS-EACA is indicated at the right of each heating curve. Wavelength of excitation, 365 mμ.

by diminished fluorescence intensity is a reflection of change in the quantum yield of fluorescence. This change may affect the lifetime of the excited state (Perrin, 1929; Pringsheim, 1949).

If γG-immunoglobulin contained DNS groups with thermally activated rotations, the ρ_h value obtained from the sucrose isotherms would reflect the contribution of the macromolecular component more accurately than the heating curves (Weber, 1952a). In Table III are given values of ρ_h calculated from the sucrose isotherms of IgG-DNS-1. Sucrose isotherms were obtained in the temperature range 5–45° and each was measured as a function of exciting wavelength. As in the case of the heating curves (Figure 1 and Table I), there is a wavelength dependence of ρ_h (see values for apparent ρ_h on the left-hand side of Table III). In addition, the ρ_h values calculated from the isotherms in sucrose increase with increasing temperature (Table III, row averages for apparent ρ_h). If the values obtained from the various isotherms are averaged at any particular wavelength, however, the apparent ρ_h varies less (Table III, column averages for apparent ρ_h). Moreover, when ρ_h is corrected by the ratio of the fluorescence intensity at each temperature to that at 25° (see Figure 5 and Discussion), the values obtained at different wavelengths and temperatures no longer show a systematic variation and range from 191 to 244 nsec (right-hand side of Table III).

Discussion

The rotational relaxation times of DNS conjugates of γG-immunoglobulins have been reported in a number of publications. Steiner and Edelhoch (1962) found values of ρ_h which ranged from 120 to 160 nsec for DNS conjugates of bovine and rabbit immuno-

TABLE III: ρ_h Calculated from Sucrose Isotherms of γG-Immunoglobulin.[a]

	Apparent ρ_h (nsec)					Corrected[b] ρ_h (nsec)					
Temp (°C)	λ_e	325	345	365	385	Row Av	λ_e	325	345	365	385	Row Av
5		119	121	163	151	139		152	155	209	193	177
10		189	159	198	202	187		234	197	246	250	232
15		170	181	174	181	177		201	214	205	214	209
20		184	175	226	244	218		201	191	246	266	226
25		180	217	258	279	234		180	217	258	279	234
30		230	184	236	255	226		212	169	217	235	208
35		271	195	246	228	235		233	168	212	196	202
40		319	265	225	343	288		258	215	182	278	233
45		284	249	273	365	293		219	192	210	281	266
Column av		216	194	222	250			210	191	221	244	

[a] Preparation is IgG-DNS-1 at 3.2×10^{-5} M (see Materials and Methods). [b] Corrected ρ_h = (apparent ρ_h)(I/I_{25}); see Figure 5. All ρ_h values calculated for water at 25°. λ_e, wavelength of excitation in millimicrons.

globulins at neutral pH. Chowdhury and Johnson (1963) reported ρ_h values for bovine immunoglobulins ranging from 76 to 93 nsec. Some of the variation in the reported values may have resulted from differences in the extent of aggregation of the samples and in the degree of contamination by free dye. Even when these variables were carefully controlled in the present study, the ρ_h values obtained from heating curves of various immunoglobulin preparations excited at 365 mμ in the absence of sucrose ranged from 93 to 131 nsec. Moreover, the value of ρ_h depended on the wavelength of excitation. The wavelength dependence of ρ_h of DNS conjugates of rabbit immunoglobulins has been reported previously (Winkler, 1963, 1965).

A second and possibly related discrepancy arises in comparing ρ_h calculated from sucrose isotherms with ρ_h calculated from heating curves in water. Wahl and Weber[2] have suggested that this discrepancy can be explained by the presence of thermally activated rotation of covalently bound DNS groups. The present experiments are compatible with this hypothesis. Addition of free DNS-EACA to DNS conjugates of γG-immunoglobulins affected the sucrose isotherm in the same way as increasing the temperature in the absence of DNS-EACA. Moreover, as will be shown below, given the data obtained from sucrose isotherms in a particular temperature range, the corresponding heating curves may be calculated from theoretical considerations. The basic assumption in this analysis is that ρ_h calculated from the sucrose isotherm reflects rotation of the macromolecule, whereas heating curves reflect both macromolecular rotation and thermally activated rotation of DNS groups.

[2] Unpublished observations referred to by Weber and Teale (1965).

Weber (1952a) has pointed out that in the presence of thermally activated intramolecular rotation or of thermally activated independent rotation of covalently bound dye, the Perrin equation may be modified as follows

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(\frac{1}{1 - f_2(T)}\right)\left(1 + \frac{3\tau}{\rho_h^{mac}}\right) \quad (5)$$

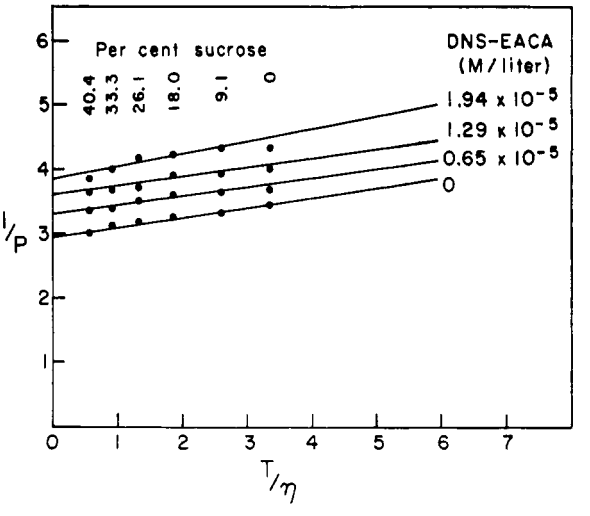

FIGURE 4: Effect of addition of DNS conjugate of ε-aminocaproic acid on polarization of a DNS conjugate of human γG-myeloma protein (Eu-DNS-2, 2.8×10^{-5} M) measured in different concentrations of sucrose at 25°. Sucrose concentrations are indicated above each set of points. DNS-EACA concentrations are indicated at the right of each isotherm. Wavelength of excitation, 365 mμ.

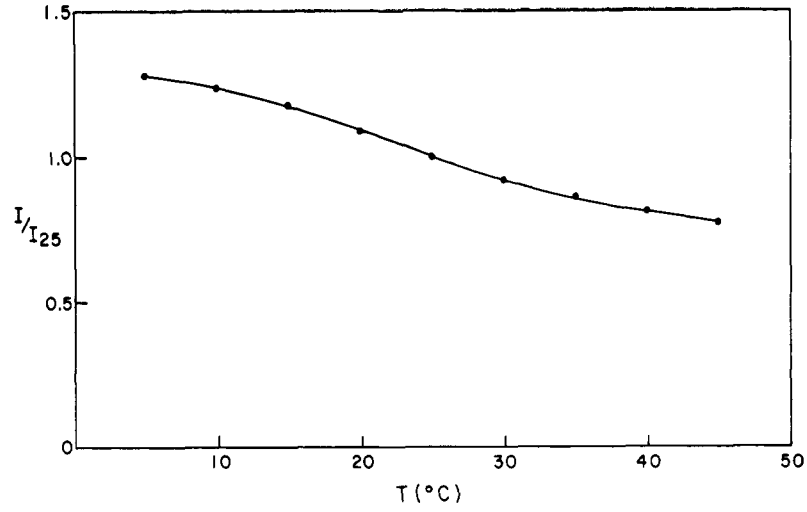

FIGURE 5: Effect of temperature on fluorescence intensity of a DNS conjugate of human γG-myeloma protein (Eu-DNS-2, 2.8 × 10^{-5} M). Results expressed as ratio of intensity at temperature (T) to intensity at 25° (I/I_{25}). Wavelength of excitation, 366 mμ; intensity of emission measured at 500 mμ.

where ρ_h^{mac} is the rotational relaxation time of the macromolecular component.

$$f_2(T) \simeq \frac{\frac{1}{p_0'} - \frac{1}{p_0}}{\frac{1}{p_0'} - \frac{1}{3}} \qquad (6)$$

$1/p_0'$ is the intercept with the ordinate axis of the tangent of the curve at a given value of T/η. $f_2(T)$ is defined as the fractional contribution of the freely rotating group to the total fluorescence intensity, when this group executes very rapid rotation independent of the slow rotation of the macromolecule (Weber, 1952a). The fractional contribution of the macromolecule to the fluorescent intensity may be defined as f^{mac}.

The conditions under which eq 5 is valid are $(\rho_2/\rho_h^{mac}) \ll 1$, $(3\tau/\rho_2) \gg 1$, and $(3\tau/\rho_2)f^{mac} \gg 1$, where ρ_2 is the rotational relaxation time of the freely rotating group. Substituting the approximate value of $f_2(T)$ in eq 5

$$\left(\frac{1}{p} - \frac{1}{3}\right) \simeq \left(\frac{1}{p_0'} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h^{mac}}\right) \qquad (7)$$

Using eq 3

$$\left(\frac{1}{p} - \frac{1}{3}\right) \simeq \left(\frac{1}{p_0'} - \frac{1}{3}\right)\left(1 + \frac{RT\tau}{\eta V^{mac}}\right) \qquad (8)$$

where V^{mac} is the effective volume of the macromolecular component. Under the conditions used in the present experiments, the sucrose isotherms showed no significant curvature, and therefore $1/p_0'$ is the intercept at the ordinate of a straight line drawn through the experimental points.

A value for ρ_h^{mac} at 25° equal to 258 nsec was computed from the sucrose isotherm at 25° using a value of τ equal to 13 nsec (Steiner and McAlister, 1957; Steiner and Edelhoch, 1962). This choice of τ appears to be reasonable inasmuch as substitution of an independently measured ρ_h^{mac} equal to 200–220 nsec (Krause and O'Konski, 1963) in eq 7 yields similar values of τ (10–12 nsec) at this temperature. Calculations of ρ_h^{mac} from sucrose isotherms at other temperatures made using the same value of τ showed a systematic increase with increasing temperature (left-hand side of Table III). If a correction (Figure 5) is introduced for possible variation in τ with quantum yield (Feofilov, 1961) the values of ρ_h^{mac} no longer exhibit a systematic variation with temperature. The average value of ρ_h^{mac} obtained from all sucrose isotherms with exciting radiation at 365 mμ was 221 nsec. This corresponds to an effective V^{mac} of 20.4 × 10^4 cm^3 $mole^{-1}$. This volume was used in eq 8; at each temperature, $1/p_0$ was taken from the corresponding isotherm (Figure 2). Assuming that V^{mac} is independent of temperature in the range from 5–45°, a value of $1/p$ was calculated for each value of T/η.

In this way, the values of $1/p$ calculated from the isotherms could be used to calculate the heating curves at different wavelengths (Figure 6) and at different sucrose concentrations at a constant wavelength (Figure 7). The agreement between the observed values of $1/p$ and those calculated at any value of T/η is good, as shown in Table IV. The observed and calculated values of $1/p_0$ and ρ_h for heating curves in the absence of sucrose are compared in this table and the error is estimated. The best fit was obtained at an

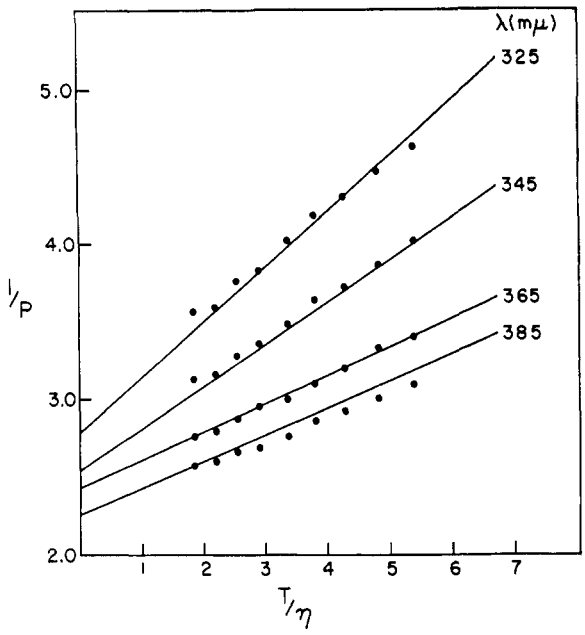

FIGURE 6: Comparison of polarizations calculated from sucrose isotherms and heating curves measured experimentally. Preparation is IgG-DNS-1 at 3.2×10^{-5} M. Wavelengths of excitation (λ) are indicated to the right of each curve. The calculated heating curves are represented by the solid lines; the points represent the experimentally obtained data.

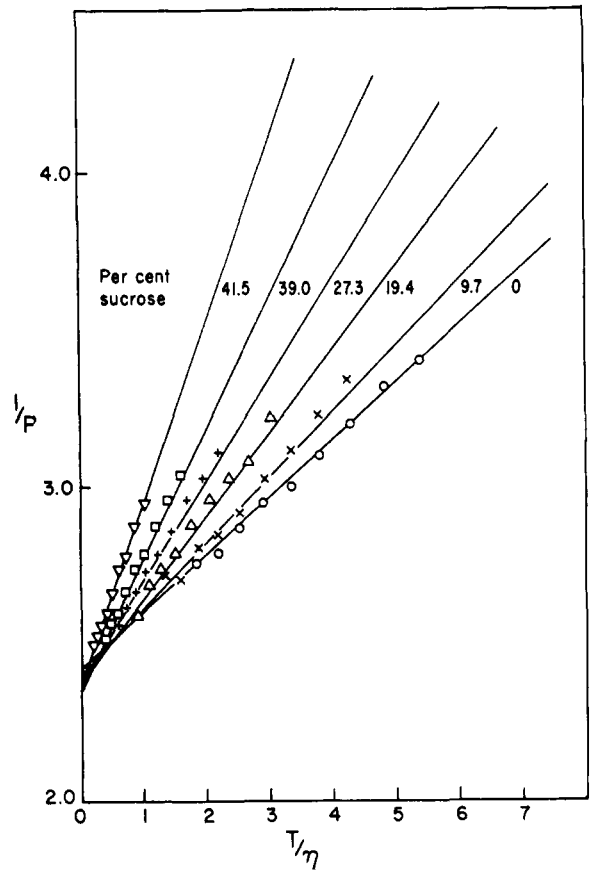

FIGURE 7: Comparison of polarizations calculated from sucrose isotherms and experimental heating curves obtained in various concentrations of sucrose. Preparation is IgG-DNS-1 at 3.2×10^{-5} M. Wavelength of excitation 365 mμ. Calculated heating curves are represented by solid lines. The different symbols refer to experimental points obtained at different sucrose concentrations. The experimental data are the same as in Figure 1.

exciting wavelength of 365 mμ (2% error) but in no case did the error in ρ_h exceed 17%. An error of 17% in ρ_h corresponds to approximately 3% error in $1/p$. In the presence of sucrose at 365 mμ (Figure 7) the agreement was within 1%.

It should be pointed out that the correction of ρ_h^{mac} for variations in quantum yield assumes that τ and quantum yield are proportional (Feofilov, 1961). Although this is the simplest assumption, its verification for this system depends upon independent measurement of τ as a function of temperature.

The agreement between the assumed model and the experimental data is consistent with the hypothesis that the heating curve anomalies result from rotation of covalently bound DNS groups. There are other lines of evidence which lend support to this hypothesis. Addition of free DNS conjugates of EACA had essentially the same effect on sucrose isotherms as raising the temperature, as predicted by eq 5. Furthermore, the ρ_h^{mac} values calculated from the sucrose isotherms in the presence of different amounts of DNS-EACA (Figure 4) were all in good agreement and averaged about 234 nsec.

The rotational relaxation times obtained from the sucrose isotherms vary slightly with wavelength of excitation, as well as with different preparations. The exact basis for the marked wavelength dependence of the heating curves is not known. It is likely to be a reflection of different environments in the vicinity of DNS groups on the γG-immunoglobulin molecule (Winkler, 1965). It is not known whether sucrose solutions interact strongly with the immunoglobulin or with the DNS groups covalently bound to its surface. Such an interaction does not appear to be responsible for the differences between the sucrose isotherms and the heating curves, inasmuch as the heating curves in the absence of sucrose may be predicted from the sucrose isotherms. Moreover, the emission spectra of DNS-IgG preparations are the same in the presence and absence of sucrose.

The value for ρ_h^{mac} of 221 nsec estimated in the present study is in excellent agreement with rotational relaxation times that have been obtained for γG-immunoglobulin by a variety of methods. A rotational relaxation time of 220 nsec was obtained from the dielectric dispersion of horse γG-immunoglobulin (Oncley, 1943). Krause and O'Konski (1963) reported a value of 200 nsec from measurements of the electric birefringence relaxation of human γG-immunoglobulin

TABLE IV: Comparison of Observed and Calculated Values of $1/p_0$ and ρ_h for Heating Curves of γG-Immunoglobulin in the Absence of Sucrose.[a]

λ_e	$1/p_0$ (obsd)	$1/p_0$ (calcd)	Rel Error in $1/p_0$	ρ_h (nsec) (obsd)	ρ_h (nsec) (calcd)	Rel Error[b] in ρ_h
325	2.92	2.78	0.05	92	80	0.15
345	2.62	2.53	0.04	103	94	0.10
365	2.41	2.42	0.01	131	133	0.02
385	2.27	2.25	0.01	151	129	0.17

[a] ρ_h calculated for water at 25°. λ_e, wavelength of excitation in millimicrons. Preparation is IgG-DNS-1 at 3.2×10^{-5} M. [b] The relative error is the absolute value of the difference between the observed and calculated $1/p_0$ or ρ_h, divided by the calculated value. Calculated values were obtained by the use of eq 8; see text.

TABLE V: Axial Ratios of γG-Immunoglobulin Calculated from ρ_h Assuming Different Degrees of Hydration.

Assumed Hydration (g of H_2O/ g of protein)	ρ_0[a] (nsec)	$\rho_h:\rho_0$	Axial Ratio[b] for Equiv Ellipsoid		$\beta \times 10^{-6}$ for Equiv Ellipsoid	
			Prolate	Oblate	Prolate	Oblate
0.0	114	1.94	5.1:1	1:4.1	2.23	2.13
0.2	146	1.51	3.0:1	1:3.0	2.16	2.13

[a] ρ_0 is the rotational relaxation time at 25° calculated for a sphere having a molecular weight of 145,000 and a partial specific volume of 0.738 (Noelken *et al.*, 1965; Marler *et al.*, 1964). ρ_h was taken to be 221 nsec. [b] Axial ratios ($a:b$) were obtained from $\rho_h:\rho_0$ by means of functions given by Weber (1953). β was obtained from the axial ratios using tables compiled by Scheraga (1961).

and computed a value of 220 nsec from the earlier flow birefringence measurements of Edsall and Foster (1948). The electric birefringence of bovine γG-immunoglobulin was measured by Ingram and Jerrard (1962) who obtained a rotational relaxation time of 215 nsec. P. Wahl and G. Weber (personal communication) have studied rabbit γG-immunoglobulin by methods similar to those of the present study and have obtained similar results. Using a pyrene conjugate of γG-immunoglobulin, Knopp and Weber (personal communication) found the rotational relaxation time to be 220 nsec. The pyrene conjugate has a longer lifetime (100 ± 6 nsec) than the DNS group and it may provide a more reliable measure of the rotational relaxation time and flexibility of the macromolecular component, particularly for very large macromolecules.

If γG-immunoglobulin is considered as a rigid ellipsoid of revolution, a range of axial ratios may be calculated from ρ_h by assuming different degrees of hydration (Table V). For a prolate ellipsoid the axial ratios range from 3 to 5. These values correspond to values of 2.16 and 2.23×10^6 for the parameter β described by Scheraga and Mandelkern (1953). A value of $\beta = 2.20 \times 10^6$ may be calculated for rabbit γG-immunoglobulin from the sedimentation and viscosity measurements of Noelken *et al.* (1965). This value is compatible with a prolate ellipsoid of revolution having an axial ratio between 3 and 4. The molecular volume of human γG-immunoglobulin may be computed given the axial ratio and ρ_h (Weber, 1953). For an axial ratio of 4 and a ρ_h of 221 nsec the volume is approximately 2.0×10^5 A^3, in agreement with other estimates (Kratky *et al.*, 1955).

A rodlike model of γG-immunoglobulin (Edelman and Gally, 1964) is consistent with data obtained in other studies of the size and shape of the molecule. The low-angle X-ray-scattering experiments of Kratky *et al.* (1955) are compatible with a cylinder of elliptical cross section with dimensions of 240 × 57 × 19 A. Electron micrographic studies (Almeida *et al.*, 1963) suggest that the molecule is extended or rodlike with a length of 220–240 A. Oncley *et al.* (1947) studied the size and shape of human γG-immunoglobulin by means of viscometry and sedimentation velocity. Assuming that the molecule was a prolate ellipsoid, they calculated a length of 235 A and a thickness of 44 A. Edsall and Foster (1948) estimated a molecular length of 230 A from streaming birefringence studies of human γG-immunoglobulin.

Although the rotational relaxation times of 200–220 nsec obtained by various methods do not exclude the possibility that the γG-immunoglobulin is some-

what flexible (Noelken *et al.*, 1965; Almeida *et al.*, 1963; Feinstein and Rowe, 1965), they are difficult to reconcile with the idea that the molecule is an extremely flexible structure. Recent studies (Metzger *et al.*, 1966) on the polarization of fluorescence of DNS conjugates of γM-immunoglobulin have led to the conclusion that different portions of that molecule can rotate independently of each other. The present analysis suggests that this conclusion should be reexamined, particularly in view of the close structural resemblance (Miller and Metzger, 1965a,b) between γG- and γM-immunoglobulins.

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