Differences in Spectral Properties and Tryptophan Content among Rabbit Anti-2,4-dinitrophenyl Antibodies of the γG-Immunoglobulin Class*

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ABSTRACT: High-affinity anti-DNP antibodies formed by rabbits late in the immune response to some dinitrophenylated proteins are more fluorescent and have a higher extinction coefficient than the low-affinity antibodies made soon after the immune response is initiated. These variations appear to arise from differences in the tryptophan content of the Fd′ piece. When the active sites of these antibodies are occupied by 2,4-dinitrophenyl (DNP) ligands virtually all of the excitation energy arising in the Fab domain, but essentially none of that arising in the Fc domain, is quenched. In these molecules, therefore, there is fair agreement between the proportion of fluorescence arising in Fab and the extent of quenching of fluorescence when the ligand binding sites are saturated (Q_{max}). In studies of some antibody–hapten reactions by the method of fluorescence quenching, the addition of less than equivalent amounts of ligand causes disproportionately more quenching than the fraction of combining sites occupied. This anomaly can be accounted for by differences in fluorescence (and in tryptophan content) of the Fab domains of diverse antidinitrophenyl molecules.

Pronounced in the heterogeneous populations of antidinitrophenyl molecules made late in the immune response, the anomalous quenching was not observed with the more homogeneous population of antibodies produced in the early stages after initiation of the immune response.

The antibody molecules made by rabbits shortly after an injection of dinitrophenylated protein have low affinity for DNP ligands, while the antibodies formed later have much higher affinity (Eisen and Siskind, 1964; Steiner and Eisen, 1967). Similar sequential changes have recently been found in the guinea pig (Nussenzweig and Benacerraf, 1967), and with some other antihapten systems in the rabbit and in the horse (Little and Eisen, 1966; Fujio and Karush, 1966; Parker *et al.*, 1967). With the anti-DNP antibodies made by rabbits the changes in affinity are observed among molecules that all belong to the γG-immunoglobulin class (Steiner and Eisen, 1967). Nevertheless, some early low-affinity anti-DNP antibodies differ from late high-affinity preparations in optical rotatory dispersion spectra and in extinction coefficient (Steiner and Lowey, 1966; Little and Donahue, 1968).

In the present study we present evidence for additional spectral and structural differences between low-affinity and high-affinity anti-DNP molecules isolated from pools of antisera obtained from groups of randomly bred rabbits at different times after the immune response is initiated. In a companion paper, some of the differences described here, as well as some additional ones, are shown to exist between the low-affinity and the high-affinity antibodies isolated from individual animals (McGuigan *et al.*, 1968).

Materials and Methods

Antigens. 2,4-Dinitrophenylated bovine γ-globulin, 2,4-dinitrophenylated hemocyanin, and 2,4-dinitrophenylated human serum albumin were prepared by treating the proteins with 2,4-dinitrobenzenesulfonic acid (Eisen, 1964). DNP-BγG[1] and DNP-hemocyanin contained 53–57 moles of 2,4-dinitrophenyl/160,000 g of protein, while DNP-HSA had 37 moles of DNP/mole of protein (70,000 g). 2,4,6-Trinitrophenylated human serum albumin, prepared according to Little and Eisen (1966), had about 35 moles of TNP/mole protein. In all these preparations the DNP and TNP groups were substituted in ε-amino groups of lysine residues (Little and Eisen, 1966; E. S. Simms, personal communication).

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[1] Abbreviations used not listed in *Biochemistry 5*, 1445 (1966), are: BγG, bovine γ-globulin; HSA, human serum albumin; TNP, 2,4,6-trinitrophenyl. Nomenclature and abbreviations for immunoglobulins and their proteolytic fragments are those recommended by the World Health Organization (*Bull. World Health Organ. 30*, 447 (1964)).

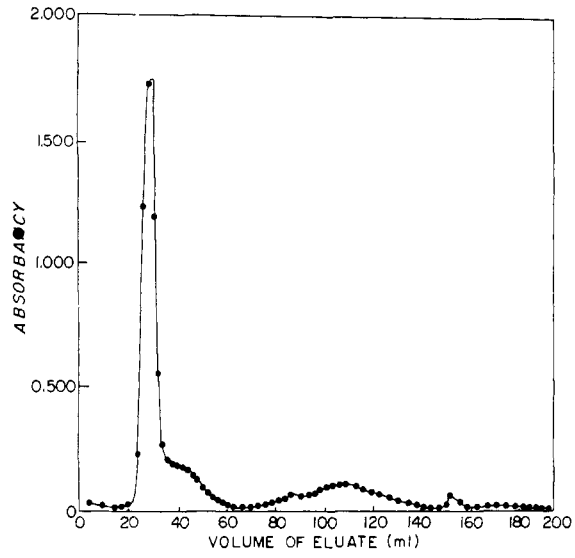


FIGURE 1: Gel filtration of pepsin digest of antibody preparation 23. Sephadex G-75 (2 × 65 cm) was equilibrated and developed with 0.002 M EDTA–0.2 M NaCl–0.01 M potassium phosphate (pH 8.2). Fractions between 20 and 35 ml were pooled ($F(ab')_2$). Absorbancy was measured at 278 mμ. The elution pattern resembles that of pepsin-digested nonspecific rabbit γG-immunoglobulin (Utsumi and Karush, 1965).

Immunization. Dinitrophenylated proteins in complete Freund's adjuvant were injected into rabbit footpads, and purified antibodies were isolated from two pools of antisera (see below for procedures). One pool (yielding purified antibody 23) represented antiserum in the early period after immunization; it was obtained from 14 rabbits that had each received 1 mg of DNP-BγG 11 days previously. The other purified antibody preparations (22A and 28) were obtained from a pool that represented antiserum in the late stages of immunization. This pool was obtained from 28 rabbits that had each received three injections of 1 mg of DNP-hemocyanin,[2] with an interval of 91 days between the first and second injections and 100 days between the second and third; the animals were bled 10 days after the third injection. All bleedings were by cardiac puncture, and sera were stored at about −20°.

Precipitin Analyses. The concentration of anti-DNP antibodies in serum was measured by the precipitin reaction. Specific precipitates, formed with DNP-HSA as antigen, were washed twice at 4° with 0.15 M NaCl, dissolved in 0.5% sodium lauryl sulfate (recrystallized from ethanol or 1-butanol), and absorbancies were determined at 278 and 360 mμ (Eisen, 1964).

Purification of Antibody. To improve yields, anti-DNP antibodies were isolated from the early and the late bleedings by modifications of the method of Farah *et al.* (1960). Antibodies were precipitated from the early pool with DNP-HSA, eluted from the precipitate with DNP-glycine, and then passed through a column containing DEAE-cellulose and Dowex 1 (Little and Eisen, 1966; Eisen *et al.*, 1967). The purified preparation thus obtained (23) is referred to hereafter as "early low-affinity antibody;" its average intrinsic association constant for the binding of 2,4-dinitroaniline was 4×10^5 M^{-1}. With the late serum pool, antibodies were precipitated with TNP-HSA, a cross-reacting antigen, eluted from the precipitate with 2,4-dinitrophenol, and subjected to ion-exchange chromatography as above; the two purified preparations (22A and 28) obtained by this procedure from aliquots of the same serum pool are hereafter referred to as "late high-affinity antibody." Each had an average intrinsic association constant for the binding of 2,4-dinitroaniline of about 2×10^8 M^{-1}. The yields of purified antibody from both the early and and the late serum pools ranged from 46 to 51% of the amount precipitated by DNP-HSA (which precipitated 90–95% as much antibody as the respective immunogens). The purified low-affinity antibody was 88% precipitable with DNP-HSA, while the purified high-affinity antibody was over 95% precipitable with the same antigen. (The precipitability of low-affinity antibodies depends on their concentration in the precipitin assay. At 1 mg/ml they are often only 80–85% specifically precipitable, but at 5 mg/ml they are usually >90% specifically precipitable; Eisen and Siskind, 1964.)

Preparation of $F(ab')_2$ and Fab' Fragments. Purified antibodies were digested with pepsin at pH 4.5 (Nisonoff, 1965), and the digests were fractionated on Sephadex G-75 (Utsumi and Karush, 1965). The first fraction to emerge from the column, $F(ab')_2$ (Figure 1), formed specific precipitates with DNP-HSA; it was also precipitated by goat antiserum specific for rabbit γG-immunoglobulin, but not by goat antiserum to the Fc fragment of rabbit γG-immunoglobulin. Reduction and alkylation of $F(ab')_2$ with 0.01 M 2-mercaptoethanol and iodoacetamide, respectively, were carried out as described by Nisnoff (1965) to yield Fab' fragments.

Preparation of Fc Fragments. Antibodies were digested with papain, and the Fc fragments were crystallized according to Porter (1959). The fragments were recrystallized three times by alternately dissolving them in 0.1 M acetate (pH 4.1) and dialyzing at 4° against 0.15 M NaCl–0.01 M potassium phosphate (pH 7.4).

Equilibrium Dialysis. Samples (1 ml) of purified antibodies (35–400 μg/ml) were dialyzed against 1.0 ml of various concentrations of [1-^{14}C]2,4-dinitroaniline, using Plexiglass chambers as available from Technilab Instruments, Los Angeles, Calif. The antibody and hapten were dissolved in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.4) and concentration equilibrium was achieved by rotating the chambers at 5 rpm for 5.5 hr at 30°. Aliquots of 0.8 ml were then removed from both the chamber with and the one without antibody, and to each was added 0.2 ml of 1 N NaOH (to denature antibody and render the hapten extractable by toluene). Thirty minutes later 16.0 ml of toluene, containing 2,5-diphenyloxazole (4 g/l.) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.1 g/l.), was added and the samples were counted in a Packard Tri-Carb liquid scintillation spec-

[2] DNP-hemocyanin was used inadvertently. However the differences in extinction coefficient, fluorescence coefficient, tryptophan content, and affinity reported here for the late antibodies formed against DNP-hemocyanin, have also been observed with the late antibodies formed against DNP-BγG (McGuigan *et al.*, 1968; see also Steiner and Lowey (1966) and Little and Donahue (1968)).

trometer. The counting efficiency for [^{14}C]toluene was 83% and the background was 25 cpm. Counting efficiency was the same over the entire range of hapten concentrations used in these studies (1×10^{-8} to 5×10^{-5} M). Correction for binding of hapten to the dialysis membranes (11–14% of the free hapten concentration) was not applied, since the contents of each chamber were counted and no volume changes were detected.[3]

Fluorescence Quenching. Fluorometric measurements of association constants were carried out in a thermostatically controlled cell in an Aminco-Bowman spectrophotofluorometer (Velick *et al.*, 1960; Eisen and McGuigan, 1968), and association constants of the antibody fragments were similarly measured by applying appropriate molecular weights (see below). Heterogeneity with respect to affinity was estimated by the Sips equation (Klotz, 1953; Nisonoff and Pressman, 1958; Karush, 1962). Computations performed with an IBM Model A360 were supported, in part, by a grant to the Washington University Computing Facilities (National Science Foundation Grant G222-96).

Tryptophan Analyses. The tryptophan content of the antibody preparations and their proteolytic fragments was measured by Little's modification of the *N*-bromosuccinimide method of Patchornik *et al.* (1960) (Little and Eisen, 1968).

Extinction Coefficients. $E^{1\%}_{1\,cm}$ of antibodies and their proteolytic fragments were determined from triplicate micro-Kjeldahl analyses of total N (Mayer, 1961), assuming 16% nitrogen, and from absorbancy measured in a Beckman spectrophotometer, Model DU.

Molecular Weights. The molecular weights used were: intact antibodies, 145,000 (Marler *et al.*, 1964); F(ab')$_2$ fragments, 92,000 (Jaquet and Cebra, 1965; Utsumi and Karush, 1965); F(ab'), 46,000; and light chains, 22,000 (Small and Lamm, 1966). For Fc we have used 60,000, the difference between 145,000 and twice the molecular weight of the Fab fragment (Jaquet and Cebra, 1965). The molecular weight values reported for Fc have, however, varied from 60,000 to 48,000 (see review by Fleischman, 1966).

[1-^{14}C]2,4-Dinitroaniline. This was prepared by a modification of the procedure described previously (Eisen and Siskind, 1964). In a sealed, glass tube 21 mg of [1-^{14}C]2,4-dinitrochlorobenzene (Nuclear Equipment Chemical Corp., Farmingdale, N. Y.) was heated with 46.4 mg of dried ammonium acetate for 63 hr at 120°. The mixture was then extracted three times with 0.01 M potassium phosphate (pH 7.4) followed by one extraction with water. The insoluble residue was dissolved in hot 95% ethanol, from which it was crystallized three times. The final product had the same melting point (184–184.5°, sintering at 155°) and the same absorption spectrum (from 280 to 440 mμ in 0.15 M NaCl–0.01 M phosphate, pH 7.4) as authentic 2,4-dinitroaniline. Under the counting conditions described (see Equilibrium Dialysis, above) the specific activity was 15.7×10^9 cpm/mmole.

Other Reagents. Hemocyanin was unfractionated serum of *Limulus polyphemus*. Bovine γ-globulin (Cohn fraction II from bovine plasma) was obtained from the Armour Pharmaceutical Co., Chicago, Ill., and crystallized human serum albumin was from Pentex, Inc., (Kankakee, Ill.). Pepsin (two-times crystallized and lyophilyzed) and twice-crystallized papain were obtained from the Sigma Chemical Co., St. Louis. Iodoacetamide was also obtained from the Sigma Chemical Co., and was recrystallized from ethanol. ε-DNP-L-lysine, from the Mann Research Laboratories (New York City), and 2,4-dinitroaniline and 2,4-dinitrotoluene, from Distillation Products Industries (Rochester, N. Y.), were recrystallized until melting points agreed with values in the literature.

Results

Binding Constants of Antibodies and Antibody Fragments. Within experimental error the early low-affinity antibody (23) and its monovalent (Fab') and bivalent (Fab')$_2$ fragments had the same affinity for several DNP ligands, and the same heterogeneity with respect to affinity (Figure 2, Tables I and II). In addition, the average intrinsic association constants were the same when measured by fluorescence quenching and by equilibrium dialysis (Table I). With the late antibody (22A) the affinity was too high to yield reliable association constants by fluorescence quenching; by equilibrium dialysis, however, this antibody and its F(ab')$_2$ and Fab' fragments had the same affinity for [1-^{14}C]2,4-dinitroaniline, and the same heterogeneity with respect to affinity (Table I). The identity of the intrinsic association constants of bivalent antibodies and their monovalent fragments means that the two ligand binding sites per rabbit γG-antibody molecule are functionally independent. These results are in accord with Nisonoff *et al.* (1959), Karush (1959), and Velick *et al.* (1960). Because the antibodies used in this study were obtained from pooled sera of several rabbits, the relatively greater homogeneity of early antibodies is not as evident here as when the antibodies are obtained from single animals (*e.g.*, Eisen and Siskind, 1964; McGuigan *et al.*, 1968).

Extinction Coefficients. The extinction coefficients at 278 mμ ($E^{1\%}_{1\,cm}$) for the early low-affinity antibody and its F(ab')$_2$ and Fc fragments were 15.9, 16.9, and 15.2, respectively. For the late high-affinity antibody and its F(ab')$_2$ and Fc fragments the values were 17.2, 18.1, and 14.3, respectively. The findings that the extinction coefficient is higher in late high-affinity anti-DNP antibody, and higher in the F(ab')$_2$ fragment than in the corresponding intact antibody, agree with those of Steiner and Lowey (1966) and Little and Donahue (1968).

Relative Fluorescence Coefficients. The fluorescence intensities of the early and late antibodies and of their proteolytic fragments were compared with a sample of pooled nonspecific rabbit γG-immunoglobulin (Table III). The emission intensity was linear with protein concentration over the range of concentrations examined (40–80 μg/ml). For a given concentration, the late anti-

[3] Volume changes were tested after equilibration by adding to occasional chambers a measured amount of DNP protein and then reading absorbancy at 360 mμ. Within experimental error (±5%) there were no detectable changes in the initial volume.

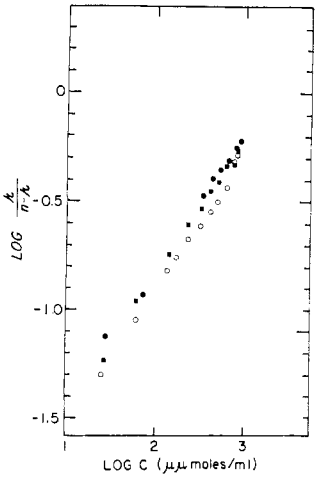
TABLE I: Average Intrinsic Association Constants for the Binding of 2,4-Dinitroaniline by Early and Late Anti-DNP Antibodies and Their Pepsin-Cleaved Fragments.[a]

Antibody		Equilibrium Dialysis		Fluorescence Quenching	
		K_0, M^{-1} ($\times 10^{-7}$)	a	K_0, M^{-1} ($\times 10^{-7}$)	a
Early preparation (23)	Intact antibody	0.042	0.6	0.044	0.6
	$F(ab')_2$ fragment	0.038	0.5	0.046	0.6
	Fab' fragment	0.036	0.6	0.038	0.6
Late preparation (22A)	Intact antibody	23	0.4		
	$F(ab')_2$ fragment	23	0.4		
	Fab' fragment	26	0.4		

[a] All measurements made in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.4) at 30°. a is the heterogeneity index with respect to K_0, the average intrinsic association constant. Fluorescence quenching values (average of duplicate titrations) were calculated with $Q_{max} = 75$ (see Figure 4). The values for antibody preparation 23 were obtained from the data of Figure 2.

body was more fluorescent, and the early antibody was about the same as nonspecific rabbit γG- immunoglobulin. The $F(ab')_2$ fragments were slightly more fluorescent than the corresponding intact antibodies. The Fc fragments from the early and the late antibodies were the same, as were those from some other rabbit γG-antibodies. These fragments were all less fluorescent (per milligram) than the intact molecules (Table III).

Relative Quantum Yields. The emission intensity at 345 mμ of several purified antibodies and of their proteolytic fragments were compared with a standard solution of tryptophan, whose fluorescence was measured immediately before that of each protein solution. The absorbance of each sample and of the tryptophan standard was also measured at 288 mμ, a λ_{max} for tryptophan. The ratio of fluorescence at 345 mμ/absorbance at 288 mμ, divided by this ratio for the tryptophan standard, was taken as the "quantum yield relative to tryptophan." The relative quantum yields were probably slightly underestimated, because about 5–10% of the absorbance of antibodies at 288 mμ is due to tyrosine, which does not fluoresce under the conditions used here (excitation was at 295 mμ and emission was recorded at 345 mμ; Weber and Teale, 1965). The relative quantum yields in fluorescence of the antibodies and their fragments was 0.23–0.30 that of free tryptophan. Thus tryptophan residues in immunoglobulins are much less fluorescent than free tryptophan, which (in water at neutral pH) has a quantum yield of fluorescence of



GURE 2: Binding of 2,4-dinitroaniline by the early low-affinity antibody (23) (■—■) and its $F(ab')_2$ (●—●) and Fab' (O—O) fragments. Fluorescence quenching was performed at 30° in 0.15 M NaCl–0.01 M phosphate (pH 7.4). Within experimental error K_0, the average intrinsic association constant, and a, the heterogeneity index (slope), were same for the intact antibody and the fragments (see Table I).

TABLE II: Average Intrinsic Association Constants for the Binding of 2,4-Dinitrotoluene and ε-DNP-L-lysine by Early Anti-DNP Antibody and Its Pepsin-Cleaved Fragments.[a]

Antibody	2,4-Dinitrotoluene		ε-DNP-L-lysine	
	K_0, M^{-1} ($\times 10^{-5}$)	a	K_0, M^{-1} ($\times 10^{-5}$)	a
Intact antibody	3.3	0.7	27	0.6
$F(ab')_2$ fragment	2.8	0.7	42	0.6
Fab' fragment	2.1	0.7	47	0.6

[a] Calculated from duplicate fluorescence-quenching titrations of preparation 23 at 30° in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.4) using $Q_{max} = 75$ (see Figure 4). a is the heterogeneity index with respect to K_0, the average intrinsic association constant.

TABLE III: Relative Fluorescence Coefficients of Rabbit Anti-DNP Antibodies and Their Fc and F(ab')$_2$ Fragments.[a]

Antibody		Fluorescence/mg per ml of Antibody or Fragment / Fluorescence/mg per ml of Nonspecific γG-Immunoglobulin
Early low affinity:	Intact antibody	0.97
	F(ab')$_2$ fragment	0.99
	Fc fragment	0.93[c]
Late high affinity[b]:	Intact antibody	1.20
	F(ab')$_2$ fragment	1.39
	Fc fragment	0.91

[a] Fluorescence was excited at about 295 mμ and emission was recorded at 345 mμ. All measurements were made at 30° in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.4) with protein at 40–80 μg/ml. Nonspecific rabbit γG-immunoglobulin was precipitated from pooled rabbit sera with 50%-saturated ammonium sulfate, followed by chromatography on DEAE-cellulose (Sober and Peterson, 1958). [b] Another preparation of antibody from the same serum pool (prepared in the same manner) had relative fluorescence coefficients of 1.05, 1.11, and 0.91 for the intact antibody, the F(ab')$_2$, and the Fc fragments, respectively. The lower values for the intact antibody and the F(ab')$_2$ fragment were probably due to quenching by contaminating traces of hapten, which are difficult to remove from preparations of late high-affinity antibodies (Eisen, 1964). [c] Fc fragments from purified rabbit antibody to the azobenzenearsonate group and from two other rabbit anti-DNP antibodies were 0.91, 0.89, and 0.93, respectively.

about 0.20 (Weber and Teale, 1965). Because the late high-affinity antibody was more fluorescent than the early low-affinity antibody (Table III), but both appeared to have about the same quantum yield in fluorescence (relative to tryptophan), their tryptophan contents were compared.

Tryptophan Content. The late high-affinity preparation had an average of 22 tryptophan residues/molecule, whereas the low-affinity preparation had 19/molecule (Table IV). Their F(ab')$_2$ fragments differed to the same extent, with 13.8 and 10.7 residues, respectively, per gmw. The Fc fragments, however, were indistinguishable with eight tryptophan residues each per fragment of 60,000 mol wt, or four per heavy chain segment. This value is in fair agreement with the three to five residues reported by Hill *et al.* (1966), who found ten tryptophan residues per Fc fragment of mol wt 57,000 and a total of six tryptophan residues in Fc peptides that accounted for 245 out of the 251 amino acid residues in Fc. The populations of light chains from the early and late antibody preparations were also indistinguishable: each had an average of two tryptophans per chain of mol wt 22,000.

The number of tryptophan residues per Fd' piece was estimated by subtracting the values for light chains from the values for F(ab')$_2$ fragments. As is shown in Table IV there were, on the average, 1.5 more tryptophan residues in the high-affinity Fd' piece than in the corresponding piece of the low-affinity preparation. The distribution of tryptophans are shown schematically in

TABLE IV: Tryptophan Content of Anti-DNP Preparations and Their Fragments.

	Moles of Tryptophan/Mole of Antibody or Fragment[a]	
	Early Low Affinity (23)	Late High Affinity (22A)
Intact antibody	19.0	22.1
F(ab')$_2$ fragment	10.7	13.8
Light chain	2.0	2.1
Fc fragment	8.2	8.2
Fd' piece (calculated)	(3.35)	(4.8)

[a] Mean values of triplicate determinations.

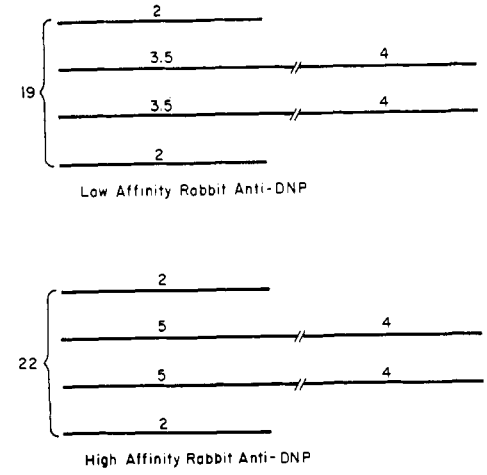


FIGURE 3: Schematic distribution of tryptophan residues in a late high-affinity antibody (22A) and in an early low-affinity antibody (23). Based on data in Table IV and Porter's (1962) model of γG-immunoglobulin.

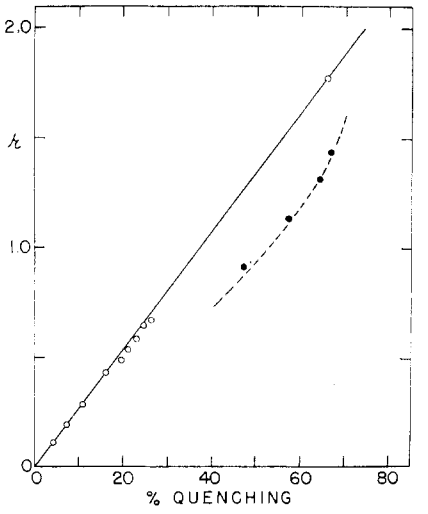


FIGURE 4: Relation between fluorescence quenching by 2,4-dinitroaniline and the average number of binding sites occupied by ligand per molecule of antibody, r. Early low-affinity antibody (O—O); late high affinity (●—●). The r values were calculated from equilibrium dialysis measurements with thesa me antibodies and [1-^{14}C]2,4-dinitroaniline. Extrapolation to $r = 2$ yields Q_{max}.

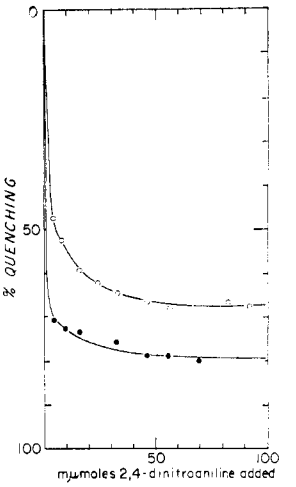


FIGURE 5: Estimation of Q_{max} by titrating antibodies with a high concentration of ligand (4×10^{-4} M 2,4-dinitroaniline). Early low-affinity antibody (O—O); late high-affinity antibody (●—●). See Table V for Q_{max} values of these antibodies and their proteolytic fragments.

Figure 3, using the four-chain structure proposed by Porter (1962).

Maximum Quenching of Antibody Fluorescence by Specifically Bound Ligand. In studies of the antibody–hapten reaction by the method of fluorescence quenching it is assumed that all the antibody molecules of a particular specificity are equally quenchable, and that quenching is linearly related to the proportion of binding sites occupied by hapten (Velick *et al.*, 1960). Because of differences in the fluorescence properties of the early and the late antibodies, it was of interest to compare their Q_{max} values, *i.e.*, the extent to which they were quenched when their ligand binding sites were saturated.

Q_{max} was determined by two methods, one suitable for low-affinity and the other for high-affinity antibodies. In the first method, the data obtained from equilibrium dialysis with a given antibody–ligand pair were used to calculate the moles of hapten bound per mole of antibody, r, for each point in the fluorescence quenching titration of the same antibody preparation with the same ligand.[4] As is shown in Figure 4, with the early low-affinity antibody quenching increased linearly with r, and extrapolation to saturation ($r = 2$, the number of ligand binding sites per molecule of γG-immunoglobulin) gave a Q_{max} value of 75. However, with the high-affinity antibody preparation quenching was not linear with r, and the extrapolated value at $r = 2$ could not be determined.

In the second method for determining Q_{max} each antibody preparation was titrated with a high concentration (4×10^{-4} M) of 2,4-dinitroaniline or ε-DNP-lysine. Corrections for nonspecific attenuation of incident and emitted light by unbound ligand gave the specific quenching curves shown in Figure 5. With the high-affinity preparation the Q_{max} was close to 80. However, the low-affinity antibody was not quite saturated by the highest level of ligand added (despite the apparent plateau; see legend, Figure 4), since the maximum quenching observed, about 65, was obtained at a total ligand concentration of 6×10^{-5} M, at which it was estimated from equilibrium dialysis that about 10% of the combining sites were still unoccupied. Saturation of the low-affinity antibody by a still higher concentration of ligand was not possible because the correction for attenuation of incident and emitted light by unbound ligand became prohibitively large. However, given the linear relationship between quenching and r for this antibody (Figure 4), the Q_{max} extrapolates to 72 at saturation.

Maximum Quenching of Antibody Fragments by Specifically Bound Ligand. As is shown in Table V, titration of proteolytic fragments with a high concentration of ε-DNP-L-lysine indicated that quenching of fluorescence of the high-affinity fragments approached 100%. With the fragments from low-affinity antibody the maximum quenching observed was only about 80%. However, these fragments had the same affinity as the corresponding intact antibodies (Tables I and II) and were thus only about 90% saturated at the highest concentration of ligand added. At saturation, therefore, they would probably have had about 90% of their fluorescence quenched. It is of interest that 2,4-dinitroaniline and ε-DNP-L-lysine quenched fluorescence almost equally well, although affinity for dinitroaniline was lower (Tables I and II).

Fluorescence Titrations of Papain- and Pepsin-Digested

[4] The following example is representative. From equilibrium dialysis with a given antibody preparation and a given ligand it is found that $r = 1.0$ at a free ligand concentration of 1×10^{-7} M. If fluorometric titration is then carried out with the same antibody at 2×10^{-8} M (1.0 ml) the concentration of bound ligand would be 2×10^{-8} M, and the free ligand concentration 1×10^{-7} M, when the total concentration of ligand is 1.2×10^{-7} M. It is thus possible to construct for a given antibody-ligand pair a curve relating total ligand added to the proportion of antibody sites occupied.

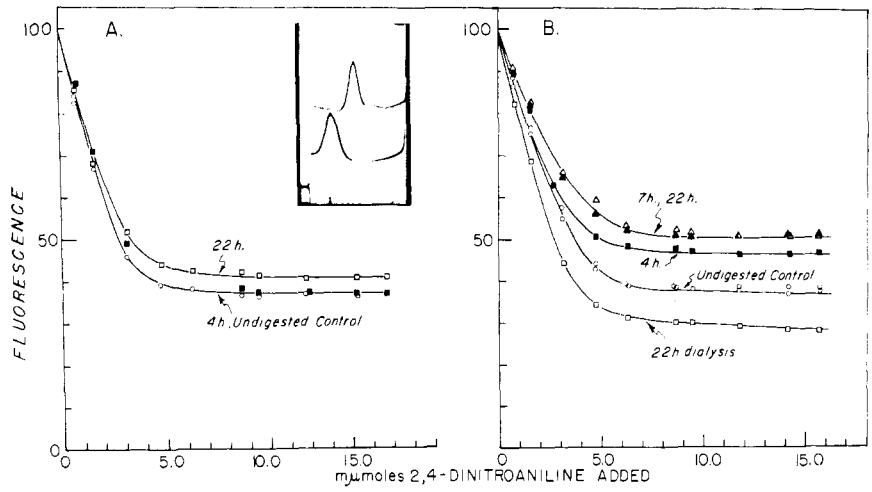


FIGURE 6: Fluorescence quenching of papain and pepsin digested anti-DNP antibodies. The antibody (32) was purified from pooled serum of 60 rabbits bled 5 weeks after an injection of 1.0 mg of DNP-hemocyanin, given 3 months after an initial injection of 1.25 mg of the same antigen. (A) Antibody (1.0 mg) was digested with 10 μg of papain at 37° in 0.01 M potassium phosphate (pH 7.3)–0.01 M cysteine–0.005 M sodium versenate (1.16 ml). Aliquots removed at 0 (O), 4 (■), and 22 (□) hr were diluted 25-fold with 0.15 M NaCl–0.01 M potassium phosphtae (pH 7.4) (buffered saline) and titrated as shown. A parallel digestion was carried out at a tenfold larger scale, and at 4 hr the digest and an undigested control were run in a Spinco Model E ultracentrifuge by Mr. Ernest Simms. In the insert photograph, taken after 64 min at 52,640 rpm, the *upper curve* is undigested control ($s_{20,w} \cong 6.5$ S, with about 1–2% faster sedimenting material, $s_{20,w} \cong 10.0$ S, as is commonly observed with lyophilized samples of γG-immunoglobulins). The *lower curve* is antibody after 4-hr digestion with papain ($s_{20,w} \cong 3.4$ S). Because of erratic temperature control S values are approximate. It is nevertheless clear that digestion by papain was complete. (B) Antibody (1.0 mg) was digested with 20 μg of pepsin at 37° in 0.01 M sodium acetate (pH 4.5) (1.01 ml). Samples withdrawn at 0, 4, 7, and 22 hr were diluted 25-fold with buffered saline and titrated as shown. After 22 hr the digest was dialyzed at 4° against 2 l. of 0.15 M NaCl–0.01 potassium phosphate (pH 7.4) and the titration was repeated on a 25-fold-diluted aliquot (22-hr dialysis).

TABLE V: Maximum Fluorescence Quenching of Anti-DNP Antibodies and Their Pepsin-Cleaved Fragments by DNP Ligands at High Concentration.[a]

Antibody		Quenching	
		ε-DNP-L-lysine	2,4-Dinitroaniline
Early low affinity	Intact antibody	65	66[b]
	$F(ab')_2$ fragment	80	73
	Fab′ fragment	78	75
Late high affinity	Intact antibody	80	80[b]
	$F(ab')_2$ fragment	>90	>90
	Fab′ fragment	>90	>90

[a] Ligand at 4.0×10^{-4} M was added in 0.01–0.03-ml increments to 1.0 ml of antibody at about 40 μg/ml (see Figure 5). Temperature, 30°; reactants in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.4). The values are those following the final addition of ligand (a total of 0.2 ml). Reduction in fluorescence was corrected for dilution, solvent blank, and absorbance by unbound ligand by comparison with a tryptophan solution to which the same amounts of the ligand were added. Thus, if the initial fluorescence of antibody and of tryptophan = 100, and the fluorescence after addition of 0.2 ml of ligand gave fluorescence values (corrected for dilution and blank) of 8 and 40, respectively, the corrected antibody fluorescence is 20 (*i.e.*, $^8/_{40} \times 100$), corresponding to quenching of 80. [b] From Figure 5.

Antibodies. The preceding results suggest that in the quenching of an anti-DNP molecule's fluorescence by specifically bound ligand the excitation energy arising in the Fab′ domain can be almost completely damped. Moreover, since Q_{max} values were close to the proportion of the antibody molecule's total fluorescence that arises in its Fab′ domains it seemed likely that none of the excitation energy arising from the Fc region was quenched by specifically bound ligand. If this were th case, and if the proteolytic fragments fluoresce as they do in intact molecules, then fluorescence-quenching titrations of whole antibodies should be superimposable on those of a papain or pepsin digest of these antibodies, with the various proteolytic fragments remaining in solution in the same proportions as they occur in intact molecules, though no longer connected by peptide

TABLE VI: Disproportionate Quenching of Late High-Affinity Antibody and Its Proteolytic Fragments by Hapten.[a]

Antibody	Total Hapten Added (as % of total antibody sites)	Quenching[b] (as % of Q_{max})
Intact antibody	14	24
	28	43
	55	72
F(ab')$_2$ fragment	16	25
	32	49
	63	70
Fab' fragment	16	22
	32	41
	63	69

[a] Fluorescence titrations were performed on preparation 22A at 30° in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.4) with 2,4-dinitroaniline. [b] Quenching is $100 - 100(F/F_0)$, where F is fluorescence after addition of ligand and F_0 the fluorescence before. The discrepancies are greater than those shown, since not all the added hapten is bound. In order to compare the intact antibody with its fragments, quenching is given here as per cent of Q_{max}, taken as 100 for the fragments and as 80 for the intact antibody (see Table V). Thus the values shown for the fragments are the actual ones observed, whereas those shown for the intact antibody are 1.25 (*i.e.*, 1/0.8) greater than the quenching actually observed.

bonds. As is shown in Figure 6, after 4-hr digestion with papain the antibody was completely degraded to fragments (~3.4 S) whose fluorescence quenching by ligand was identical with that of the intact antibody. After 22-hr incubation, however, the digest was slightly less quenchable, suggesting a slowly developing change in the Fab or in the Fc fragments (see pepsin below).

In contrast to papain, digestion with pepsin led, even after 4 hr, to decreased fluorescence quenching (Figure 6), although the extent of specific binding was unaltered (see Tables I and II). However, after removal of some of the pepsin fragments by dialysis the remaining material was quenched to an even greater extent than the intact antibody. Thus some of the small pepsin fragments (*e.g.*, those that can be removed by dialysis) probably fluoresce to a greater extent than they do when they are integral components of intact antibody molecules. This result would account, at least in part, for the difference in fluorometric titration of the intact antibody and its pepsin digest.

Anomalous Quenching. In fluorescence-quenching titrations of high-affinity anti-DNP antibodies it is often observed that the addition of less than saturating amounts of ligand quenches a larger proportion of the antibody's fluorescence than can possibly be accounted for by the proportion of antibody sites occupied (Eisen, 1964). This anomaly, which is not observed with low-affinity anti-DNP molecules, was seen in the titration of the late antibody but not the early antibody (Figure 4). The disproportionality with the late high-affinity antibody is illustrated by the representative data shown in Table VI, in which quenching is expressed as percentage of Q_{max} in order to simplify comparison of intact antibody with fragments having different Q_{max} values (80 for the high-affinity antibody, and about 100 for its fragments). These data are discussed below.

Discussion

The low-affinity anti-DNP antibodies made within 2 weeks after injecting rabbits with certain dinitrophenylated proteins have lower extinction coefficients and are less fluorescent than the high-affinity molecules synthesized later. The differences can be accounted for qualitatively by the finding of fewer tryptophan residues per molecule, on the average, in the early low-affinity antibodies. The observation that low-affinity molecules differ in amino acid composition from high-affinity molecules of the same specificity (anti-DNP) and same immunoglobulin class (γG) has been substantiated by more extensive analyses of antibodies isolated from individual rabbits at various times after immunization (McGuigan *et al.*, 1968). The localization of the additional tryptophan residues of high-affinity molecules in the Fd' piece is in accord with the rule that amino acid sequences in N-terminal halves of chains differ from one immunoglobulin to another, while sequences in the C-terminal halves are uniform for chains of a given immunoglobulin class (Hilschmann and Craig, 1965; Titani *et al.*, 1965; Piggot and Press, 1967).

Noelken *et al.* (1966) proposed a model for the rabbit γG-immunoglobulin molecule in which its three domains (2 Fab plus 1 Fc) are essentially independent globular regions connected by short lengths of loosely coiled polypeptide chain. The present findings are consistent with this model. Thus, the fluorescence quenching of papain-digested antibody, in which the several proteolytic fragments were all present in solution, can be essentially identical with that of the intact antibody, showing that excitation energy arising in one domain of the intact molecule is not transferred to another. Similar results were previously obtained with rabbit anti-TNP antibodies (Little and Eisen, 1966). In addition, the isolated Fab' (and F(ab')$_2$) fragments from both the early and the late antibody had the same affinity for DNP ligands, and the same heterogeneity with respect to affinity, as the corresponding intact antibodies, showing that these domains function independently; *i.e.*, that there are probably no significant interactions between the two active sites for a particular ligand in a given γG-antibody molecule.

The relative fluorescence coefficients shown in Table III suggest that the isolated proteolytic fragments fluoresce essentially as they do in intact antibody molecules. For example, the value for the high-affinity antibody, 1.20, is close to the sum of the fluorescence coefficients

of its F(ab')$_2$ and Fc fragments, adjusted for their molecular weights ($0.65 \times 1.4 + 0.35 \times 0.9$). The proportion of the whole molecule's fluorescence which arises in the Fab' domains thus appears to be 0.75 and 0.67 for the high-affinity and the low-affinity preparations, respectively. These values are close to the Q_{max} values observed when the respective antibodies are saturated by ligand (75–80 and 70–75, respectively). This agreement likewise supports the view, expressed above, that when the fluorescence of a rabbit anti-DNP antibody is quenched by specifically bound ligand almost all the excitation energy arising in the Fab domain, but none of that arising in the Fc domain, is damped. In contrast to rabbit anti-DNP, the Q_{max} for rabbit anti-TNP antibodies is only 50–60 (Little and Eisen, 1966, 1968). The difference between anti-TNP and anti-DNP (in the the rabbit) is due to their respective Fab fragments: those from anti-TNP molecules are less fluorescent, and their transfer of excitation energy to bound ligand is less efficient, than those from anti-DNP molecules (Little and Eisen, 1968).

Because the combining sites are active in Fab' fragments it is possible to exclude one mechanism for the anomalous quenching of fluorescence frequently observed with preparations of high-affinity anti-DNP antibodies. As shown in Table VI, for example, when the amount of hapten added to such an antibody preparation corresponded to 14, 28, and 55% of the total antibody sites, fluorescence was quenched 24, 43, and 72%, respectively, of the maximal quenching observed as saturation. Since an amount of hapten that corresponded to 55% of the total antibody combining sites caused 72% of maximal quenching, the addition of sufficient hapten to saturate the remaining 45% of sites can cause only 28% quenching. This means, if we assume that the two combining sites per antibody (γG) molecule are identical, that about one-half the molecules make twice the contribution of the other half to fluorescence quenching (*e.g.*, $^{72}/_{55}$ *vs.* $^{28}/_{45}$). Comparable results were observed with the F(ab')$_2$ fragments. These discrepancies could arise if the binding of a ligand molecule to one active site were to quench both the fluorescence originating in the corresponding Fab domain as well as some of the fluorescence arising in the distant domain, containing the other binding site of the same bivalent antibody molecule or fragment. However, this possibility is excluded by the observation that the same anomaly is encountered with univalent Fab' fragments (Table VI).

While a variety of other possibilities may be entertained, we prefer the following explanation, which is suggested by the present findings. Populations of high-affinity anti-DNP molecules obtained late in immunization are heterogeneous not only with respect to affinity but also with respect to their fluorescence properties. The molecules of higher than average affinity are probably more fluorescent because they contain more tryptophan. Thus, when limiting amounts of ligand are added, and are bound preferentially to the antibodies of higher than average affinity, they tend also to be bound preferentially to the more fluorescent molecules, causing disproportionately more damping of fluorescence than the fraction of total sites occupied.

In considering this suggestion it should be kept in mind that high-affinity anti-DNP differs from low-affinity anti-DNP not only in having some more tryptophan residues, but probably also in being slightly more quenched at saturation (*cf.* Q_{max} of 70–75 for antibody 23 and 75–80 for antibody 22A, and the comparable differences in Q_{max} of their Fab fragments, given in Table V).[5] Thus, the approximately two-fold greater quenching of the more readily saturated molecules, shown in Table VI, does not require that they have as much as twice the amount of tryptophan in their Fab fragments.

The heterogeneity of anti-DNP molecules with respect to tryptophan content and fluorescence thus limits the accuracy of fluorescence quenching as a procedure for measuring intrinsic association constants and their heterogeneity. The limitation applies particularly to populations of molecules of high average affinity, which are especially heterogeneous (Eisen and Siskind, 1964). Indeed, because of their heterogeneity with respect to tryptophan fluorescence, measurement of their average intrinsic association constant by fluorescence quenching would be expected to exaggerate estimates of their heterogeneity with respect to affinity for ligand (see Figures 4 and 5 in Little and Eisen, 1966). This limitation is negligible, however, with populations of anti-DNP molecules of low average affinity: as shown elsewhere (Eisen and Siskind, 1964; see also McGuigan *et al.*, 1968), they are less heterogeneous with respect to affinity, and they probably are also less heterogeneous with respect to fluorescence coefficient and tryptophan content (see Figure 4). The early, low-affinity antibodies obtained from individual rabbits may also have considerably fewer light chain bands in polyacrylamide disc electrophoresis than late, high-affinity antibodies isolated from the same animals (Cohen and Dresser, 1966; Eisen *et al.*, 1967).

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[5] Quenchability is not necessarily proportional to the number of tryptophan residues in Fab, *e.g.*, rabbit anti-TNP antibodies (of the γG class) are quenched only about 55% when saturated by ligand, even when these antibodies have the same tryptophan content as rabbit anti-DNP, whose Q_{max} is about 75 (Little and Eisen, 1968).

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