Physical and Chemical Differences between Rabbit Antibodies to the 2,4-Dinitrophenyl and the 2,4,6-Trinitrophenyl Groups*

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ABSTRACT: Rabbit antibodies prepared against the 2,4-dinitrophenyl (DNP) group were compared with those formed against the 2,4,6-trinitrophenyl (TNP) group with respect to ultraviolet absorbance and fluorescence spectra and tryptophan content. Despite similar binding constants for homologous ligands and extensive cross-reactivity, purified preparations of the two antibodies were consistently different in (a) the maximum extent of quenching of their fluorescence by specifically bound ligands (Q_{max}), (b) their fluorescence intensities, and (c) the increase in fluorescence following denaturation in guanidine. The differences between the intact antibodies were also exhibited by their active (Fab) fragments.

The tryptophan content varied among different preparations, but was greater than in nonspecific rabbit γG-immunoglobulins. Though the range of values overlapped, tryptophan tended to be more abundant in populations of anti-DNP than in populations of anti-TNP molecules. The differences in fluorescence between the two antibodies, and between their active fragments, are in accord with a higher tryptophan content and higher fluorescence yield per tryptophan residue of anti-DNP molecules.

Rabbit antibodies to the 2,4-dinitrophenyl (DNP)[1] group and to the 2,4,6-trinitrophenyl (TNP) group cross-react extensively and share many physical, chemical, and immunological properties. They have the same range of binding constants with homologous ligands and similar degrees of binding constant heterogeneity, and the most extensively examined preparations are γG-immunoglobulin molecules with uncorrected sedimentation constants of 6.3–6.7 S. However, during the study of hapten binding by the method of fluorescence quenching it was found that rabbit anti-TNP antibodies are less quenchable than anti-DNP antibodies when their respective active sites are saturated (*i.e.*, Q_{max} is smaller for anti-TNP than for anti-DNP), and that this difference is a property of the antibody molecules, not the ligands (Little and Eisen, 1966).[2] The difference in fluorescence quenching has led us to compare the amino acid composition and the ultraviolet absorbance and fluorescence spectra of anti-DNP and anti-TNP antibodies. The present paper reports differences in tryptophan content, in ultraviolet extinction coefficients, in alkaline spectra, and in fluorescence spectra between diverse populations of these two rabbit antibodies. The data support the view that anti-TNP and anti-DNP molecules differ in primary structure and that the differences are localized in those molecular domains (Fab) that encompass the active sites.

Materials and Methods

Purified Antibodies and Immunoglobulins. The methods employed for the production, quantitation, and isolation of anti-TNP and anti-DNP antibodies were the same as those described previously (Little and Eisen, 1966; Eisen *et al.*, 1968). Each lot of purified antibodies showed a single precipitin arc of γG mobility on immunoelectrophoresis developed with goat antiserum to a crude rabbit globulin fraction, and each was more than 90% precipitable with a homologous hapten–protein conjugate (HSA bearing 36–37 moles of DNP or of TNP per mole of albumin). Rabbit antibodies specific for the *p*-azophenylarsonate group were isolated as described (Lark *et al.*, 1965), using a modification of the method of Koshland *et al.* (1962). Most antibodies

* From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110. *Received October 13, 1967.* This work was supported, in part, by Research Grants AI-03231 and AI-52742 and Training Grant 5TI-AI-257 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and by a contract (DA-49-193-MD-2330) with the Research and Development Command, Department of the Army, recommended by the Commission on Immunization of the Armed Forces Epidemiological Board.

† Part of this work was performed during tenure of a special fellowship award from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (5F3 AI-18, 1210-02).

[1] Abbreviations and nomenclature used: TNP, the 2,4,6-trinitrophenyl group; DNP, the 2,4-dinitrophenyl group; HSA, human serum albumin; BγG, bovine γ-globulin; NBS, *N*-bromosuccinimide. The antipolynitrophenyl antibodies formed against 2,4-dinitrophenylated proteins and those formed against 2,4,6-trinitrophenylated proteins cross-react extensively. They are referred to as anti-DNP antibodies and anti-TNP antibodies, respectively, because each consistently forms more stable complexes with homologous ligands (J. R. Little and H. N. Eisen, to be published). The immunoglobulin nomenclature and abbreviations used are those recommended by the World Health Organization (*Bull. World Health Organ. 30*, 447, 1964).

[2] Q_{max} is the percentage quenching of antibody fluorescence when ligand binding sites are saturated.

were obtained from randomly bred albino rabbits. Antibodies of known immunoglobulin allotype were isolated from race A rabbits, which were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine.[3]

Nonspecific rabbit γG-immunoglobulins were isolated by the method of Levy and Sober (1960). These preparations also yielded a single arc of γG mobility in immunoelectrophoresis, using a goat antiserum to a crude rabbit globulin fraction to develop precipitin bands.

Proteolytic Cleavage of Antibody. Papain and pepsin digestion were carried out at pH 7.5 and 4.5, respectively, under the conditions described by Nisonoff (1964). Papain fragments were resolved on CM-cellulose at pH 5.4, after removal of the crystalline fraction that separated from the reaction mixture during dialysis at 4° against 0.01 M phosphate (pH 7.4) (Porter, 1959). The bivalent 5S antibody fragment obtained by pepsin digestion was separated from smaller peptides on Sephadex G-75 under the conditions described by Utsumi and Karush (1965).

Reduction, Alkylation, and Separation of Light and Heavy Chains. The conditions for mild reduction and alkylation of antibodies were those of Fleischman *et al.* (1962). Following alkylation with iodoacetamide the reaction mixture was applied directly to a 2.4 × 50 cm column of Sephadex G-100 equilibrated with 1 M propionic acid and 4.5 M urea, and eluted with the same solvent. Recovery of optical density units (278 mμ) varied between 90 and 100% and the pooled light chain fractions amounted to 26–30% of the total absorbance recovered.

Alkaline Spectra. Absorbance spectra of proteins in alkali were obtained by the method of Bencze and Schmid (1957) in a Cary Model 14 recording spectrophotometer, except that all protein solutions were first dissolved in 6.4 M guanidine hydrochloride at pH 7.4 for 2 hr at 0–4° and then adjusted to 0.1 M NaOH. Final protein concentrations were 0.4 to 0.6 mg/ml.

Tryptophan Analyses. Tryptophan was measured by procedure K of Spies and Chambers (1949) scaled down tenfold in volume, and by a modification of the *N*-bromosuccinimide (NBS) method (Ramachandran and Witkop, 1959). Each protein was dissolved in 6.3 M guanidine hydrochloride–0.1 M acetate (pH 4) and 2 hr later NBS titrations were carried out at protein concentrations between 100 and 500 μg/ml in a Beckman Model DU or a Zeiss PMQ II spectrophotometer. Analyses were carried out without thermostatic control. Protein concentrations were determined from ultraviolet absorbance at 278 mμ in neutral aqueous solution using the values determined for $E^{1\%}_{1\,cm}$ (see below) and a molecular weight of 145,000 (Lamm and Small, 1966).

Kjeldahl Nitrogen. Samples of protein estimated to contain 50–100 μg of N were clarified by centrifugation and replicate absorbance measurements were obtained in a Zeiss PMQ II spectrophotometer in 0.15 M NaCl–0.02 M phosphate (pH 7.4) (buffered sample). Aliquots (1 ml) were digested for 3 hr in concentrated H_2SO_4 using a copper catalyst (Mayer, 1961). Ammonia was distilled in a micro steam distillation apparatus from Fisher Scientific Co., using low N reagent NaOH (obtained from Hartman Leddon Co., Philadelphia, Pa.). Distillate (40 ml) was collected as a single aliquot in saturated boric acid. Titrations with 0.0143 N HCl were performed with a mercury calibrated microburet and a Corning Model 12 pH meter for end-point determination. Replicate samples agreed to within ±3%. $E^{1\%}_{1\,cm}$ values were calculated from Kjeldahl N, assuming 16% N in rabbit γG-immunoglobulins and antibodies.

Fluorescence Measurements. Fluorescence was measured in an Aminco–Bowman spectrophotofluorometer with a temperature-controlled cell housing that maintained the sample temperature within ±0.2°. The emission monochromator was calibrated with a mercury discharge lamp, and intensities were read only after the 5–10 min necessary for the sample temperature to stabilize at 30°. Readings were corrected by subtracting the fluorescence of the solvent, which was usually 2–4% of the protein fluorescence. Association constants for the binding of ligands by antibodies were determined from fluorescence quenching titrations as described (Little and Eisen, 1966; Eisen, 1964).

Relative fluorescence coefficients, defined as the emission intensity of protein divided by the protein concentration, were determined from fluorescence intensity at 345 mμ, with excitation at 290 mμ, using arbitrary but fixed amplifier settings. The effect of guanidine denaturation on fluorescence was measured by diluting aliquots of native antibodies 1:10 in buffered saline and in the same buffer with 6.7 M guanidine hydrochloride. Repeated measurements over several hours after dilution into guanidine or buffer showed only a 1–2% decline in fluorescence.

Other Reagents. Guanidine hydrochloride was obtained from Distillation Products Industries (Rochester, N. Y.) and was recrystallized from methanol after treatment with powdered charcoal (Norit A). Additional reagents were used without further purification. 2-Mercaptoethanol was obtained from Distillation Products Industries (Rochester, N. Y.) and iodoacetamide and *N*-bromosuccinimide were the products of Fisher Scientific Co. (St. Louis, Mo.). Papain, pepsin, and crystallized tryptophan were obtained from the Sigma Chemical Co. (St. Louis, Mo.). DEAE-cellulose (0.51 mequiv/g) and CM-cellulose (0.68 mequiv/g) were Serva resins from Gallard-Schlesinger Chemical Manufacturing Co. (Garden City, N. Y.).

Results

Comparison of Q_{max} Values. The Q_{max} values for several lots of purified antibodies and their proteolytic

[3] We wish to thank Dr. Sheldon Dray for determining the allotypes of the rabbit antibodies and for pointing out that race A rabbits have a high incidence of homozygosity for some of the light and heavy chain allotypes (1, 3, and 4).

TABLE I: Q_{max} Values and Binding Constants for Antibodies and Antibody Fragments.[a]

Antibody or Antibody Fragment	Ligand	Q_{max}	Av Intrinsic Assoc Constant (K_0) ($M^{-1} \times 10^{-7}$)
Anti-TNP			
TN-9	ε-TNP-aminocaproate	56	>10
TN-9	2,4-Dinitroaniline	56	3.3
TN-9	2,4,6-Trinitrotoluene	53	1.4
TN-9	2,4-Dinitrotoluene	54	0.90
TN-9 $F(ab')_2$	ε-TNP-L-lysine	81	>10
TN-9 Fab 1	ε-TNP-aminocaproate	72	>10
TN-9 Fab 11	ε-TNP-aminocaproate	72	>10
TN-8	ε-TNP-aminocaproate	56	4.6
TN-0 (5 weeks)	ε-TNP-aminocaproate	52	>10
TN-0 (10 weeks)	ε-TNP-aminocaproate	61	>10
TN-5	ε-TNP-aminocaproate	56	0.21
Aa 3-3, Ab 4-4, No. 704	ε-TNP-aminocaproate	52	2.3
Aa 3-3, Ab 4-4, No. 411	ε-TNP-aminocaproate	56	1.9
Aa 1-3, Ab 4-4, No. 423	ε-TNP-aminocaproate	52	4.1
Aa 1-3, Ab 4-4, No. 600	ε-TNP-aminocaproate	58	2.2
Average for intact anti-TNP antibodies		55	
Anti-DNP			
DN-22	ε-DNP-L-lysine	70	>10
DN-22 $F(ab')_2$	ε-DNP-L-lysine	92	>10
DN-22 Fab 1	ε-DNP-aminocaproate	88	>10
DN-22 Fab 11	ε-DNP-aminocaproate	85	>10
DN-7A	2,4-Dinitroaniline	72	>10[b]
DN-31 (P1)	ε-DNP-aminocaproate	75	>10
DN-31 (P5)	ε-DNP-aminocaproate	70	>10
Aa 3-3, Ab 4-4, No. 857	ε-DNP-aminocaproate	75	>10
Aa 3-3, Ab 4-4, No. 855	ε-DNP-aminocaproate	75	>10
Aa 1-3, Ab 4-4, No. 472	ε-DNP-aminocaproate	72	>10
Aa 1-3, Ab 4-4, No. 473	ε-DNP-aminocaproate	72	>10
Average for intact anti-DNP antibodies		73	

[a] The Q_{max} values were obtained by the method of Day (1963) using antibody fluorescence quenching titrations with high ligand concentrations ($4–5 \times 10^{-4}$ M) (see text). Corresponding titrations of tryptophan (as the free amino acid) with the same ligand at the same concentration were used to correct for attenuation of fluorescence by the high concentration of unbound ligand. Separate aliquots of each antibody population were also titrated by fluorescence quenching using a much lower concentration of the same ligand ($2–4 \times 10^{-6}$ M) in order to calculate the average intrinsic association constant (K_0). All the immunoglobulins listed were isolated from pools of sera from randomly bred rabbits, except those with the indicated light-chain and heavy-chain allotypes which were obtained from individual animals (listed by number in the table). Antiserum pool DN-31 was fractionated by sequential addition of small amounts of antigen (Eisen and Siskind, 1964); P1 and P5 represent the isolated anti-DNP antibodies from the first and fifth fractions obtained from this serum pool. [b] Estimated from data in Eisen and Siskind (1964).

fragments are summarized in Table I and illustrated in Figure 1. The values were obtained by titrating antibody samples (40–60 μg/ml) with a highly concentrated solution of a strongly bound ligand (*e.g.*, $4.0–5.0 \times 10^{-4}$ M ε-DNP- or ε-TNP-aminocaproate) and correcting for absorbance of incident and emitted light by unbound ligand (Day, 1963). The correction was based on the decrease in fluorescence of a standard solution of tryptophan to which the same ligand was added (see legend, Figure 1).

The Q_{max} values obtained in this manner are only accurate for antibody–ligand pairs that interact strongly (high affinity), since the method requires saturation of the antibody's binding sites. At the concentrations of ligand ($\sim 4 \times 10^{-4}$ M) and antibody ($\sim 2 \times 10^{-7}$ M) usually employed, about 50% of the combining sites

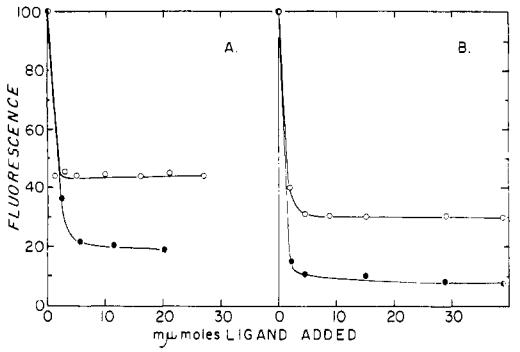

FIGURE 1: Q_{max} values for a preparation of anti-TNP antibodies and their active fragments in A and for a preparation of anti-DNP antibodies and their active fragments in B. Purified antibody (1 ml, 60 μg/ml) or the corresponding F(ab')$_2$ fragment (40 μg/ml) was titrated with a concentrated solution of hapten. Changes in fluorescence intensity after additions of hapten were corrected for absorbance due to the high concentration of unbound ligand. The corrected fluorescence quenching curves are shown in A for the titration of anti-TNP antibodies (TN-9) with 5×10^{-4} M ε-TNP-aminocaproate (○——○) and of F(ab')$_2$ fragments from TN-9 with 5×10^{-4} M ε-TNP-L-lysine (●——●). In B are shown analogous quenching curves for titrations with 4.6×10^{-4} M ε-DNP-L-lysine of anti-DNP antibodies (DN-22) (○—○) and F(ab')$_2$ fragments from DN-22 (●——●). The Q_{max} values calculated from these data are given in Table I.

TABLE II: Relative Fluorescence Coefficients of Antibodies and Fragments.[a]

Antibody	Concn (μg/ml)[b]	Fluorescence (345 mμ)	Rel Fluorescence Coefficient (fluorescence/0.1 mg ml^{-1})
Anti-TNP			
TN-5 (TNP-OH eluted)	88	7.5	85[c]
TN-5 (DNP-OH eluted)	109	10.0	92[c]
704 (TNP-OH eluted)	56	41.2	74
704 (DNP-OH eluted)	56	41.4	74
TN-9	42	29.6	71
Anti-DNP			
DN-14	318	32.4	102[c]
DN-8	154	20.0	130[c]
DN-31	229	32.0	140[c]
855 (TNP-OH eluted)	54	76.6	142
855 (DNP-OH eluted)	55	75.5	137
DN-22	53	64.4	121
Fab Fragments from			
TN-9	39	30.4	77
DN-22	36	100	274

[a] Fluorescence intensities of all samples were measured at 30° in arbitrary units. Values are corrected for the blank (2–4%) due to the solvent, which was 0.15 M NaCl–0.02 M phosphate (pH 7.4). Antibodies 704 and 855 were isolated from sera of the individual rabbits of known allotype (Aa 3-3, Ab 4-4) noted in Figure 2 and in Tables III–V. These sera were each divided into aliquots from which antibodies were isolated with either homologous or cross-reacting antigen and hapten (see text). DNP-OH and TNP-OH are 2,4-dinitrophenol and 2,4,6-trinitrophenol. [b] Based on absorbance at 278 mμ and the extinction coefficients of Table V. For TN-5 the extinction coefficient used was 15.8, the average of anti-TNP antibodies (Table V), and for DN-8 and DN-31 the value used was 16.4, the average of anti-DNP antibodies, omitting one early antibody preparation of low affinity (DN-5). [c] Because protein concentrations of these samples were high, the fluorescence intensities were recorded at a tenfold lower amplifier gain. The fluorescence values (column 3) were multiplied by ten in obtaining relative fluorescence coefficients for these samples in order to express all derived values (column 4) in terms of the same amplifier gain.

with association constants of 1.5×10^4 M^{-1}, and a larger proportion of those with lower association constants, would be expected to remain unoccupied at the last point on the titration curve.

In agreement with other observations the average Q_{max} value for anti-TNP preparations was 55 (Little and Eisen, 1966) and for anti-DNP preparations was 73 (Eisen and Siskind, 1964; J. E. McGuigan and H. N. Eisen, in preparation). Q_{max} values were much higher with Fab and F(ab')$_2$ fragments than with intact molecules, and were higher with the fragments from anti-DNP than with those from anti-TNP molecules.

The Q_{max} values of anti-DNP preparations also agree with the proportion of the total fluorescence emanating from their Fab domains (J. E. McGuigan and H. N. Eisen, in preparation), and with the evidence, given below, that essentially all the excitation energy of the Fab domain, but none from the Fc domain, is quenched by bound DNP or TNP ligands. In addition, the agreement between binding constants determined by (1) equilibrium dialysis and (2) fluorescence quenching with the empirically determined Q_{max} provides confidence in the values obtained (Little and Eisen, 1966). Antibodies isolated from different antisera had slightly different Q_{max} values, but the range of values for each specificity was narrow. Purified antibodies isolated independently from different aliquots of the same antiserum had essentially the same Q_{max}. Representative differences between high affinity antibody preparations and their respective Fab fragments are shown in Figure 1.

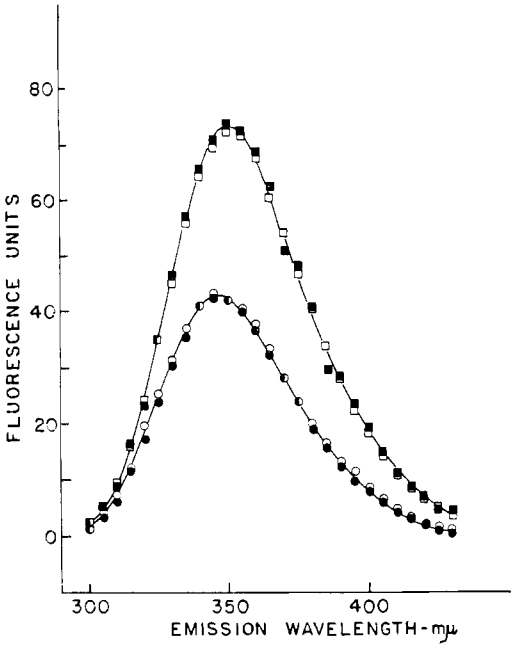

FIGURE 2: Antibody fluorescence emission spectra. Each protein sample had an absorbance of 0.088–0.090 at 278 mμ in buffered saline, and each fluorescence emission was obtained with excitation at 295 mμ (30°). The anti-DNP antibody preparations (squares) were isolated from separate aliquots of a single antiserum (855), using DNP reagents for specific precipitation and hapten elution (■——■) with one aliquot and TNP reagents for the other (□——□). The anti-TNP antibody preparations (circles) were also isolated from separate aliquots of a single antiserum (704), using TNP reagents (○——○) for purification from one aliquot and DNP reagents (●——●) for the other. The emission λ_{max} for both anti-DNP preparations was 350 mμ and for both anti-TNP preparations was 345 mμ. Each antiserum (855 and 704) was obtained from a different rabbit with the Aa3-3, Ab4-4 allotype. The nonspecific γG-immunoglobulins isolated from preimmunization bleedings of each animal had indistinguishable fluorescence intensities for samples with the same absorbance at 278 mμ.

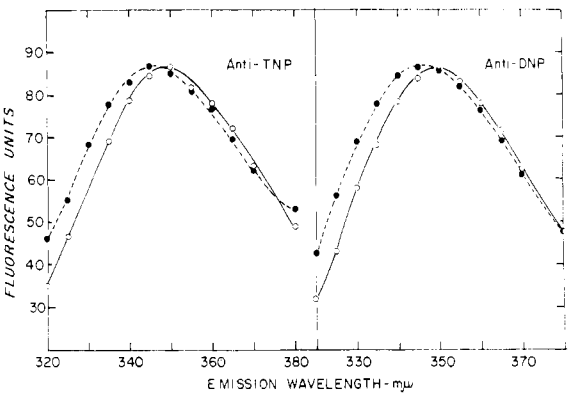

FIGURE 3: Fluorescence shift due to ligand binding. Samples (1 ml) of anti-TNP (TN-9) and anti-DNP (DN-22) antibodies (55 μg/ml in buffered saline) were equilibrated at 30° and emission spectra (○——○) were obtained with excitation at 295 mμ. The same two antibody preparations were then mixed with their homologous aminocaproate haptens (6×10^{-6} M) and emission spectra were again obtained (●– – –●). The spectra are normalized to the maximum intensity of the anti-DNP preparation before hapten addition. Ligand binding sites were essentially saturated since both antibody preparations had association constants for the homologous aminocaproate haptens $>1 \times 10^{8}$ M^{-1}. Controls with nonspecific rabbit γG-immunoglobulin showed no shift with addition of DNP- or TNP-aminocaproate.

Relative Fluorescence Coefficients and Emission Spectra. Relative fluorescence coefficients for representative antibodies from randomly bred and inbred animals are given in Table II. Values for anti-DNP preparations were consistently higher, and no overlap with the values for anti-TNP antibodies was observed.

The fluorescence excitation spectra of anti-TNP and anti-DNP molecules were indistinguishable, but their emission spectra were different (Figure 2). To examine the possibility that the differences resulted from unequal contamination with bound ligands, pairs of purified antibody samples were isolated from the respective antisera (*i.e.*, one from an anti-DNP and one from an anti-TNP antiserum) using identical reagents for specific precipitation and hapten elution. Because of extensive cross-reactivity, anti-DNP molecules could be recovered in high yield from anti-DNP sera using 2,4,6-trinitrophenol to elute the antibodies from specific precipitates made with TNP proteins; conversely, anti-TNP antibodies could be isolated through the use of DNP proteins to precipitate and dinitrophenol to elute the antibodies from the precipitates. Each antiserum was thus divided into two aliquots and antibodies were purified from one with DNP reagents and from the other with TNP reagents. The fluorescence emission spectra of the four antibody populations are shown in Figure 2. The fluorescence intensity was significantly greater for both anti-DNP preparations. If ligand molecules retained in the antibody combining sites were responsible for the difference one would expect less fluorescence in the antibody purified with homologous reagents rather than the cross-reacting ones. In addition, absorbancy at 340–360 mμ, where the haptens absorb maximally, indicated that only 1–6 mole % of antibody combining sites were occupied by ligand after the isolation procedures employed here (Eisen *et al.*, 1967; Little and Eisen, 1966). Contamination by hapten was approximately equal for the anti-DNP and the anti-TNP antibodies. Since retained hapten quenches anti-DNP more than anti-TNP (see the difference in Q_{max} above), it is clear that the differences observed in relative fluorescence

TABLE III: Increase in Immunoglobulin Fluorescence with Denaturation by Guanidine Hydrochloride.[a]

γG-Immunoglobulin	Fluorescence Intensity (F) at 345 mμ: Native (N) (buffered saline)	Denatured (D) (6.0 M guanidine·HCl)	F(D): F(N)
	Anti-TNP		
TN-5 (R2)	10.0	28.2	2.82
TN-5 (RRT)	8.2	30.9	3.77
TN-5 (WW)	7.5	20.9	2.79
TN-2	11.7	52.2	4.46
TN-0	18.7	75.2	4.02
704	25.9	85.2	3.29
		Anti-TNP av	3.52
704 Fab fragment	21.2	46.9	2.21
	Anti-DNP		
DN-14	32.5	72.3	2.22
DN-8	20.1	40.1	1.99
DN-31 (P4)	22.5	61.5	2.73
DN-31 (PP)	32.1	61.6	1.92
855	43.6	87.8	2.01
		Anti-DNP av	2.17
855 Fab fragment	22.9	40.8	1.78
	Anti-*p*-azophenylarsonate		
R-Azo	29.1	68.1	2.34
	Nonspecific γG		
704 (preimmunization)	34.9	79.4	2.27
855 (preimmunization)	36.8	88.4	2.40

[a] One-volume aliquots of a stock solution of antibody or γG-immunoglobulin in 0.001 M potassium phosphate (pH 7.6) were diluted into nine volumes of buffered saline and separately into nine volumes of 6.7 M guanidine·HCl in buffered saline. The fluorescence intensity of the two diluted aliquots was then measured consecutively at 30° without changing amplifier settings between sample readings. Fluorescence measurements were corrected for small solvent blanks. Since each aliquot of a given pair contained the same amount of immunoglobulin, no correction was made for the 10–15% decrease in absorbance (278 mμ) of each sample in guanidine when compared with its partner in buffered saline. Excitation was at 290 mμ and emission at 345 mμ. All antibody samples were isolated from antiserum pools except for rabbits 704 and 855 whose allotypes are noted in Figure 2 and Tables IV and V. Antisera TN-5 and DN-31 were fractionated by sequential addition of small amounts of antigen (Eisen and Siskind, 1964); three fractions of the TN-5 (R2, RRT, and WW) and two fractions of DN-31 (P4 and PP) were examined.

coefficients and in antibody fluorescence emission spectra are minimal estimates. Similar differences in fluorescence emission spectra were obtained between aliquots (with equal absorbance at 278 mμ) of anti-TNP and anti-DNP antibodies isolated from pools of randomly bred rabbits.

Figure 3 shows the blue shift in the emission spectra that followed saturation of the active sites of both anti-DNP and anti-TNP antibodies. Each of the antibodies shown had an average binding constant with the homologous aminocaproate derivative which was too high to be measured accurately by fluorescence quenching ($>1 \times 10^8$ M^{-1}). Since the concentration of total ligand used in this experiment was 8.3×10^{-7} M and the concentration of antibody sites was about 4.8×10^{-7} M, the ligand binding sites were essentially saturated. When the same concentrations of DNP- or TNP-aminocaproate were added to tryptophan or to nonspecific γG-immunoglobulin, there were no shifts in emission spectra. Because transfer of excitation energy appears to be confined to the Fab domain (Little and Eisen, 1966; J. E. McGuigan and H. N. Eisen, in preparation), the residual fluorescence, shifted to lower wavelength, probably corresponds approximately to the emission spectrum of the Fc domain.

Denaturation in Guanidine Hydrochloride. The increase in antibody fluorescence with denaturation is shown in Table III. When equal aliquots of antibody solution were diluted in buffered saline and in the same buffer containing 6.7 M guanidine hydrochloride the fluorescence intensity (345 mμ) was greater in the guanidine-denaturated protein. The increase in fluorescence achieved a stable maximum within minutes in 6.0 M guanidine hydrochloride. The increment was consistently greater with anti-TNP than with anti-DNP antibodies and nonspecific γG-immunoglobulins (Table III). The increase was also greater for an anti-TNP Fab fragment than for the Fab fragment of an anti-DNP antibody, suggesting that the difference observed with the intact molecules originated in the active fragment.

Tryptophan Analyses. Tryptophan values, expressed as residues per molecule of protein, are shown in Table IV for samples of anti-DNP and anti-TNP antibodies of low, intermediate, and high affinity, as well as for antibodies from individual rabbits homozygous at the known heavy- and light-chain loci. The values found by the NBS method were statistically indistinguishable from those measured by the method of Spies and Chambers ($P \gg 0.05$; for statistical analysis, see Snedecor, 1937). The values for anti-DNP and anti-TNP samples overlapped to such an extent that with many preparations it was not possible to distinguish the two kinds of antibodies on the basis of tryptophan content. Nevertheless, some anti-DNP preparations, especially those with high affinity, obtained after prolonged immunization, had distinctly high levels. Moreover, anti-TNP samples, taken as a group, differed significantly with respect to tryptophan from anti-DNP samples, also taken as a group (P value for the comparison of the two groups by analysis of variance was $\ll 0.01$ (Snedecor, 1937)). Despite the

TABLE IV: Tryptophan Content of Rabbit γG Antibodies and Nonspecific γG-Immunoglobulins.[a]

γG-Immunoglobulins	Days after Immunization	Av Tryptophan Residues/Molecule of Protein: *N*-Bromosuccinimide Method	Av Tryptophan Residues/Molecule of Protein: Method of Spies and Chambers
	Anti-TNP		
TN-13	13	16.5 ± 0.1	17.4 ± 0.2
TN-8	16	20.0 ± 1.5	19.1 ± 0.3
TN-9	386 (boosted)	20.6 ± 0.3	18.2 ± 0.2
704	42	19.3 ± 0.3	
	Anti-DNP		
DN-23	11	19.5 ± 0.1	
DN-14	43	20.5 ± 0.3	20.9 ± 0.3
DN-22	190 (boosted)	23.2 ± 0.2	24.4 ± 0.2
855	42	25.1 ± 0.3	
	Nonspecific γG[b]		
Aa 3-3, Ab 4-4		16.6 ± 0.2	
D2		17.8 ± 0.2	17.0 ± 0.1

[a] All the immunoglobulins listed were isolated from pools of sera from randomly bred rabbits except for 704 and 855 which were from individual donors of the same allotypes (3,3/4,4). One of the samples of nonspecific γG was a pool from two rabbits of the same allotype (Aa 3-3, Ab 4-4). Calculations were based on the extinction coefficients shown in Table V and a molecular weight of 145,000. Values are means plus and minus standard deviation for triplicate analyses. [b] These values are about 10–15% lower than those reported by Crumpton and Wilkinson (1963) and by Smith *et al.* (1955). By an ultraviolet absorbance method, the former found 2.90 g of tryptophan/100 g of nonspecific rabbit γ-globulin, while the latter, using the Spies and Chambers method, found 2.70 g/100 g of protein for four rabbit antibodies to diverse pneumococcal polysaccharides. These values correspond, respectively, to 20.6 and 19.2 residues per protein molecule of 145,000 mol wt.

wide range of values the over-all difference is probably valid because the anti-TNP and anti-DNP samples in Table IV were obtained from comparable sera (*e.g.*, TN-9 and DN-22 represented late bleedings, TN-13 and DN-23 represented early ones, and the antibodies from rabbits 704 and 855 were each obtained 42 days after immunization with TNP-Bγ G and DNP-Bγ G, respectively). It is also notable that there was more tryptophan in anti-DNP antibodies, and possibly more also in anti-TNP, than in nonspecific rabbit γG-immunoglobulins.

Antibody and γG-Immunoglobulin Extinction Coefficients. Table V shows that the extinction coefficients for anti-TNP and anti-DNP antibodies were higher than those of nonspecific γG-immunoglobulins. With the anti-DNP antibodies there was a tendency for a progressive increase in $E^{1\%}_{1\,cm}$ with increasing antibody affinity. Some of the samples of Table V were also examined by Steiner and Lowey (1966), whose data are in agreement. Preparations of purified antibodies fractionated by eluting antibodies from a specific precipitate first with 2,4,6-trinitrophenol and then with TNP-aminocaproate had extinction coefficients that were not notably different.

Alkaline Spectra. Anti-TNP and anti-DNP antibodies also differed in the relative height of the absorbance peaks attributed to tryptophan (about 280 mμ) and ionized tyrosine (about 293 mμ) in alkaline solution Because the development of stable spectral patterns in 0.1 M NaOH requires many hours, the antibody samples were preincubated in 6.4 M guanidine hydrochloride for 2 hr before exposure to 0.1 M NaOH. Stable alkaline spectra were then obtained in a few minutes. Comparison of alkaline spectra for three antibody preparations and their respective light and heavy chains yielded the patterns shown in Figure 4. The results are consistent with a higher tryptophan: tyrosine ratio in some anti-DNP molecules (Table IV) and they indicate that these differences are present in the corresponding heavy chains. Alkaline spectra of light chains from anti-DNP and anti-TNP molecules were indistinguishable.

Discussion

In the present study the main differences found between rabbit anti-TNP and anti-DNP molecules of the γG-immunoglobulin class are as follows. (1) Anti-TNP molecules have a lower Q_{max} than anti-DNP molecules (Figure 1 and Table I). (2) Anti-TNP molecules are less fluorescent than anti-DNP molecules (Table III). (3) The relatively diminished fluorescence of anti-TNP molecules appears to arise primarily from the lower quantum yield of their tryptophan residues.

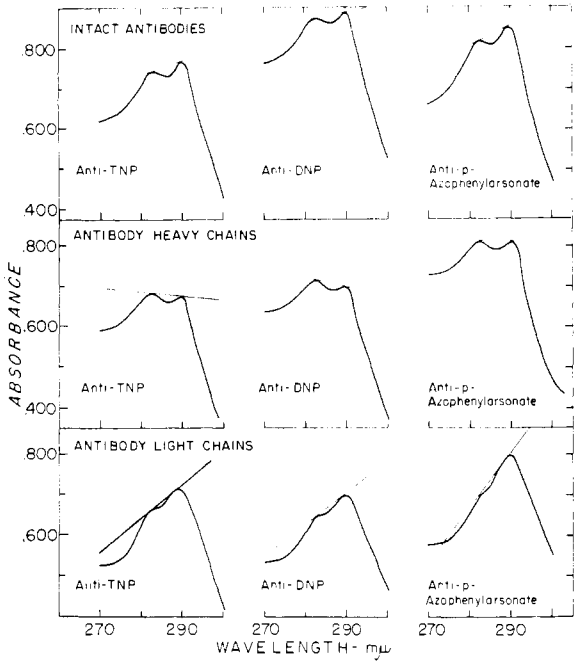

FIGURE 4: Alkaline absorbance spectra of antibody molecules and their respective heavy and light chains. Anti-TNP (TN-9), anti-DNP (DN-14), and anti-*p*-azophenylarsonate antibodies (500 μg/ml in 0.001 M potassium phosphate, pH 7.6) were incubated in 6.4 M guanidine hydrochloride for 2 hr before exposure to 0.1 M NaOH. The three spectra in the upper panel were obtained within 15 min after addition of alkali. The light and heavy chains (500 μg/ml) were dialyzed overnight against 6.4 M guanidine hydrochloride and then exposed to 0.1 M NaOH. Tangents were drawn through the two λ_{max} of each alkaline spectrum and the normalized slopes were calculated according to Bencze and Schmid (1957). The values for intact anti-TNP, anti-DNP, and anti-*p*-azophenylarsonate antibodies were 4.6, 2.4, and 6.0, respectively; for the corresponding heavy chains the values were −1.2, −2.5, and 0; for the corresponding light chains the values were 12, 13, and 20.

TABLE V: Affinity and Extinction Coefficient of Rabbit γG-Immunoglobulins.[a]

γG-Immunoglobulin	$E^{1\%}_{1\,cm}$ 278 mμ	Av Intrinsic Assocn Constant (K_0) ($M^{-1} \times 10^{-7}$)[b]
	Anti-TNP	
TN-13 (TNP-OH eluted)	16.0	1.1
TN-13 (ϵ-TNP-aminocaproate eluted)	16.0 ± 0.2	
TN-8	14.9 ± 0.1	4.7
TN-7	16.2 ± 0.1	2.6
TN-9	15.8 ± 0.2	>10
704	15.3	2.2
	Anti-DNP	
DN-5	14.5 ± 0.2	0.014
DN-23	15.9 ± 0.2	0.48
DN-14	16.0 ± 0.5	>10
DN-22	16.8 ± 0.2	>10
855	16.2 ± 0.5	>10
	Nonspecific γG	
D-2	14.6 ± 0.4	
Aa 3-3, Ab 4-4	14.7 ± 0.4	

[a] The preparations of immunoglobulins are the same as those in Table II except for antibodies from rabbit serum pools TN-7 and DN-5, obtained 50 and 10 days after immunization, respectively. The two preparations of antibody lot TN-13 were obtained by specific precipitation with TNP-HSA and then sequential elution of the washed specific precipitate with 0.01 M 2,4,6-trinitrophenol (TNP-OH eluted) and 0.001 M ϵ-TNP-aminocaproate (ϵ-TNP-aminocaproate eluted). The extinction coefficients were based on micro-Kjeldahl analysis; the values are means plus and minus standard deviation. Where no standard deviations are given, the means were obtained from duplicate analyses. [b] Association constants were determined for anti-TNP antibodies with ϵ-TNP-L-lysine and for anti-DNP antibodies with ϵ-DNP-L-lysine.

In addition, anti-DNP antibodies, especially those of high affinity, from late bleedings, tend to have more tryptophan residues per molecule (Table IV; also J. E. McGuigan and H. N. Eisen, in preparation). (4) The differences in Q_{max} and in fluorescence between the intact antibody molecules are also exhibited by their respective Fab fragments (Figure 1 and Tables I and II).

These findings provide a qualitatively coherent explanation for the different changes in fluorescence that occur when anti-DNP and anti-TNP antibodies are titrated with DNP or TNP ligands. As mentioned above, the transfer of excitation energy appears to occur only within the Fab domains of anti-TNP (Little and Eisen, 1966) and anti-DNP (J. E. McGuigan and H. N. Eisen, in preparation) rabbit antibodies of the γG-immunoglobulin class. The Fc portions of these antibodies should have the same fluorescence properties since they are cocrystallizable and the amino acid sequence of this part of all rabbit γG-immunoglobulin molecules appears to be the same (Hill *et al.*, 1966). If the isolated Fab fragments have the same fluorescence properties as the corresponding domains of the intact γG molecules, then the fluorescence emanating from Fab domains would be lower in anti-TNP than in anti-DNP molecules. Accordingly, with saturation of ligand binding sites and nearly complete quenching

of Fab fluorescence, the residual fluorescence, due mostly to Fc, would be greater in anti-TNP, *i.e.*, saturated anti-TNP would be less quenched than saturated anti-DNP. This difference would also be expected if either DNP or TNP ligands were used to saturate either type of antibody, and this expectation has been verified (Little and Eisen, 1966).

Two findings suggest that there is a lower quantum yield of fluorescence of the tryptophan residues in anti-TNP than in anti-DNP molecules. First, in preparations that have the same tryptophan content, the anti-TNP molecules were less fluorescent (*e.g.*, TN-9 and DN-14 in Tables II and IV). Second, the increase in fluorescence following denaturation is greater with anti-TNP than with anti-DNP. Like most globular proteins, anti-DNP and anti-TNP antibodies show a significant increase in fluorescence upon denaturation (Table III). Guanidine was chosen as the denaturing agent in the present study because Buckley *et al.* (1963) and Noelken and Tanford (1964) have shown by optical rotatory dispersion that rabbit antibodies, antibody fragments, and nonspecific γG-immunoglobulins are effectively unfolded by this reagent. Teale (1960) has shown that despite great variations in the quantum efficiency of tryptophan fluorescence in various native proteins, they display essentially the same quantum efficiency per tryptophan residue (0.23 ± 0.03) in 8 M urea. The greater increase in the fluorescence of anti-TNP molecules upon denaturation thus suggests that there is a lower quantum yield of fluorescence for the tryptophan residues of native anti-TNP antibodies as compared with native anti-DNP or nonspecific γG-immunoglobulins.

The alkaline spectrum of a protein reflects its content of tryptophan and tyrosine (for review, see Light and Smith, 1963). However, we have been reluctant to use these spectra to estimate tryptophan content, for several reasons: *e.g.*, some destruction of tryptophan occurs in 0.1 N NaOH (Spies and Chambers, 1949), and the exact positions of the alkaline spectral maxima for tryptophan (near 280 mμ) and for ionized tyrosine (near 293 mμ) differ in different proteins (Beaven and Holiday, 1952). Because of the latter difficulty, Bencze and Schmid (1957) suggested the use of the slope of a line drawn tangent to the two maxima to obtain a mole ratio of tryptophan to tyrosine. As shown in Figure 4, the tangents for representative antibody samples indicate that the tryptophan:tyrosine ratios tend to be higher in anti-DNP molecules. Furthermore, the alkaline spectra of the heavy chains isolated from the anti-DNP and anti-TNP preparations differed in the same way as the corresponding intact antibody molecules, whereas the isolated light chain alkaline spectra were indistinguishable. Since Fc fragments of diverse rabbit γG-immunoglobulins seem to be identical (Hill *et al.*, 1966) these results suggest that the difference between the tryptophan:tyrosine ratio of anti-DNP and anti-TNP populations arise from differences in the Fd piece. These findings are consistent with evidence for the location in this region of heavy chains of the variable amino acid sequences that presumably specify the active sites of antibodies (Press *et al.*, 1966; Porter and Weir, 1966). In contrast, our preparation of rabbit anti-*p*-azophenylarsonate antibodies had a relatively low tryptophan:tyrosine ratio (Figure 4), and the low ratio was evident in both its light and heavy chains. These findings are in accord with (1) the relatively high tyrosine content of other preparations of rabbit anti-*p*-azophenylarsonate antibodies (Koshland *et al.*, 1964), and (2) the distribution of the excess tyrosine residues in both the light and heavy chains isolated from these antibodies (Koshland *et al.*, 1966). The extent to which light chains participate in forming the active sites of antibodies is still uncertain (see Fleischman (1966) for review).

The spectral and analytical data presented here indicate that anti-DNP and anti-TNP molecules differ in primary structure despite their many similarities and their extensive cross-reaction. Since the structural differences appear to be localized in the Fd piece of the heavy chain, our findings are consistent with other evidence that isolated heavy chains retain the ability to bind ligands selectively (Fleischman *et al.*, 1963; Utsumi and Karush, 1965; Haber and Richards, 1966).

Acknowledgment

We are indebted to Helaine Donahue for skillful technical assistance.

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The Effects of Actinomycin D on the Biosynthesis of Plasma Lipoproteins*

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ABSTRACT: Intraperitoneal administration of actinomycin D, particularly when supplementary injections were given, severely inhibited orotic acid incorporation into the trichloroacetic acid precipitate of rat liver for several hours and resulted in a decrease in the principal low density lipoprotein component of rat serum, which was still apparent after 21 hr. At the same time the concentration of high density lipoproteins was found to increase slightly. The biosynthesis of lipoproteins was measured by following L-[^{3}H]lysine incorporation into the lipoprotein protein moieties, both *in vivo* and in the isolated perfused liver.

In both cases actinomycin D pretreatment caused a greater inhibition of low density lipoprotein synthesis than that of high density lipoprotein. Specific activity determinations on a number of protein fractions isolated from the perfusate and from the liver after a liver perfusion demonstrated that the decreases in synthesis observed after actinomycin D treatment vary considerably for different proteins, indicating a general heterogeneity of hepatic template stability.

Evidence has been presented that mRNA of significantly prolonged stability exists in reticulocytes, a highly differentiated mammalian tissue essentially synthesizing a single type of protein (Marks *et al.*, 1962). As an organ displaying a wide variety of enzymatic functions and synthesizing a number of different proteins for export, the liver might be expected to exhibit a heterogeneous stability of its mRNA population. Pitot *et al.* (1965) have presented evidence that the templates coding for several liver enzymes have quite different lifetimes. A number of investigators have measured hepatic protein synthesis after blocking or inhibiting RNA synthesis with actinomycin D (Singer and Leder, 1966). Recently John and Miller (1966) measured the synthesis of the specific plasma proteins, albumin and fibrinogen, in a perfused liver system and found the synthesis of the latter molecular species to be more sensitive to inhibition by actinomycin D.

With the exception of the chylomicrons, serum lipoproteins are synthesized mainly, if not exclusively, by the liver (Roheim *et al.*, 1967). Two species of

* From the Department of Biochemistry, University of Florida College of Medicine, Gainesville, Florida 32601. *Received September 20, 1967.* Supported by National Institutes of Health Research Grant AM 02457 and Florida Heart Association Grant 67-AG-5. Taken in part from a dissertation submitted by G. R. F. to the University of Florida in partial fulfillment of the requirements of the degree of Doctor of Philosophy, Dec 1966.

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