Fluorescent Probes for the Study of the Antibody–Hapten Reaction. II. Variation in the Antibody Combining Site during the Immune Response*

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ABSTRACT: Fluorescence enhancement was evaluated as a quantitative system for the study of rabbit antibodies specific for the 5-dimethylaminonaphthalene-1-sulfonyl (DNS) group. Following immunization progressive increases occurred with time in the affinity of anti-DNS antibody for ε-DNS-lysine. The fluorescence spectrum of the bound dye also changed. In successive bleedings obtained during the first several weeks after immunization the fluorescence quantum yield of bound dye increased progressively. Late in the immune response, however, a fall in quantum yield sometimes occurred. Fluorescence enhancement also was used as a means of screening for differences in the microenvironment of the combining sites of anti-DNS antibodies. Rabbit anti-DNS antibody from individual animals was fractionated on DEAE-cellulose into two populations, both γG-globulins. The emission maximum of ε-DNS-lysine bound to the more positively charged antibody fraction (that eluted first from the DEAE-cellulose) was generally in the 496–501-mμ range.

The emission maximum of dye bound by the more negatively charged antibody fraction usually was in the 502–505-mμ range. A possible explanation for this difference is that the binding sites in the two DEAE fractions have different conservative regions. However, alternative possibilities also must be considered.

One of the characteristic features of the immune response is a marked variation in the affinity of the antibody combining site for homologous ligand. The systematic evaluation of factors in the immunization program which influence antibody affinity has been facilitated to a considerable extent by the technique of fluorescence quenching of purified antibody which was introduced by Velick *et al.* (1960). For the most part fluorescence quenching studies have utilized antibody specific for the 2,4-dinitrophenyl (DNP) group. When ε-DNP-lysine is bound by purified rabbit anti-DNP antibody, as much as 70–80% of the antibody fluorescence can be quenched. Quenching occurs by means of excitation energy transfer from excited aromatic amino acid residues of the protein to the ligand. Fluorescence quenching provides a means of rapidly evaluating antibody affinity with the expenditure of 40 μg or less of purified anti-DNP antibody. Utilizing fluorescence quenching Eisen and Siskind (1964) demonstrated that in rabbits immunized with DNP-bovine globulin in adjuvant the average affinity of antibody for ε-DNP-lysine rose progressively from about 10^5 mole^{-1} at 10 days to about 10^8 mole^{-1} at 2 months after immunization. Fluorescence quenching also has been valuable in establishing that the specificity of anti-DNP antibodies can extend beyond the ε-DNP-lysine moiety to neighboring amino acids in the immunizing antigen (Eisen *et al.*, 1964; Parker *et al.*, 1966; Counts and Little, 1966; Paul *et al.*, 1966).

One drawback to the fluorescence quenching method is a requirement for antibody with a high degree of purity. The specific purification of anti-DNP antibody involves precipitation (or absorption) with specific antigen followed by hapten elution. Substantial amounts of antibody are lost during purification and the characterization of the antibody is limited to only a portion of the original antibody population. While there is no evidence to indicate that the antibodies which are not recovered differ substantially from those which are, it would be desirable if the entire antibody population could be titrated. In principle this goal could be met by utilizing changes in hapten fluorescence on binding to homologous antibody as a measure of the antibody–hapten reaction. A binding system which might be suitable was described in the companion paper (Parker *et al.*, 1967) where it was shown that antibodies specific for the 5-dimethylaminonaphthalene-1-sulfonyl (DNS)[1]

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[1] Abbreviations used: DNS, the 5-dimethylaminonaphthalene-1-sulfonyl group; HSA, human serum albumin; phosphate–saline, 0.15 M NaCl–0.01 M phosphate (0.008 M dipotassium phosphate and 0.002 M monosodium phosphate), pH 7.4; Ff (340-480) is the fluorescence of free hapten (ε-DNS-lysine) in arbitrary units on activation at 340 mμ, and measurement of fluorescence at 480 mμ; Fb (340–480) is the corresponding value for antibody-bound hapten (ε-DNS-lysine); Fb/Ff (340–480) is the ratio of the two; r is the ratio of moles of hapten bound

group markedly increased the fluorescence of ε-DNS-lysine. In the present paper fluorescence enhancement has been evaluated as a quantitative system for the study of anti-DNS antibodies.

Material and Methods

Limulus hemocyanin was a gift of Dr. H. N. Eisen. The following materials were obtained commercially: human serum albumin and rabbit serum albumin (Pentex, Inc., Kankakee, Ill.), bovine γ-globulin (Armour & Co., Chicago, Ill.), and Dowex 1-X8 and diethylaminoethylcellulose (lot 16B-2380) (Sigma Chemical Company, St. Louis, Mo.).

DNS–protein conjugates were prepared as described by Weber (1952). Conjugates were purified by extensive dialysis against dilute sodium bicarbonate. The absence of unconjugated DNS derivatives in the final product was verified by chromatography on Sephadex G-25 in PO_4–saline (Pharmacia Fine Chemicals, Piscataway, N. J.). The extent of substitution of proteins by DNS groups was estimated from the protein dry weight and the absorbance at 330 mμ, after a correction for contributions by the protein moiety. Protein DNS-lysyl residues were taken to have a molar absorbancy of 4.57×10^3 (see Parker *et al.*, 1967). ε-DNS-lysine and DNS-sulfonate were prepared as described in the companion paper (Parker *et al.*, 1967).

Random bred white rabbits were immunized with 2 mg of DNS–protein in a volume of 1.6 ml in complete Freund's adjuvant distributed among the footpads as described previously (Parker *et al.*, 1966). While the immune response to a number of different DNS–protein conjugates (DNS–human serum albumin, DNS–bovine γ-globulin, and DNS–hemocyanin) was studied the majority of the observations were with sera from animals immunized with DNS_{360}–hemocyanin. Animals were bled before immunization and at intervals thereafter. Sera were obtained by cardiac puncture and stored at −20° until use.

Globulin fractions were prepared by several different methods: (1) precipitation (twice) with ammonium sulfate at 40% of saturation; the precipitates were washed three times with ice-cold 40% ammonium sulfate; (2) precipitation (twice) with ammonium sulfate at 33% of saturation followed by three washes with 33% saturated ammonium sulfate; (3) precipitation with decreasing concentrations of sodium sulfate as described by Kekwick (1940); and (4) by preliminary precipitation with $(NH_4)_2SO_4$ at 40% of saturation followed by chromatography on DEAE-cellulose.

Quantitative precipitin analysis was performed as described by Eisen (1964a). Washed precipitates were dissolved in 0.5% sodium lauryl sulfate (Matheson Coleman and Bell, Norwood, Ohio) and read at 280 and 340 mμ in the spectrophotometer. The 280-mμ absorbancy of rabbit anti-DNS antibody was taken to be 1.5 (1-cm light path) (see below). A correction was made for contributions by antigen to the 280-mμ reading. Immunoelectrophoresis was carried out according to the procedure of Scheidigger (1955) using goat antirabbit γ-globulin and sheep antirabbit serum (Hyland Laboratories, Los Angeles, Calif.) as antisera.

Specifically purified antibody was isolated by specific precipitation (method I) or by specific absorption (method II). In both instances the antibody was separated from antigen by elution with hapten.

Method I. In a typical purification 30 ml of serum containing 30 mg of rabbit anti-DNS_{360}–hemocyanin antibody was incubated with the equivalent amount of DNS_{30}–HSA (6 mg) at 37° for 1 hr and 4° for 16 hr. The precipitate was washed two times with ice-cold saline and two times with saline at room temperature. The washed precipitate was incubated with 7.5 ml of 0.18 M 1-dimethylaminonaphthalene-5-sulfonate (DNS-sulfonate; Weber, 1952) in 0.02 M PO_4 (pH 7.5) at 37° for 30 min. The supernatant was isolated by centrifugation and passed through a DEAE Dowex-1 column (69 ml of DEAE, wet volume, equilibrated with 0.02 M PO_4 (pH 7.0) on top of 34 ml of Dowex 1-X8, wet volume, equilibrated with 0.15 M NaCl–0.01 M phosphate (pH 7.4)). The column was eluted with 0.02 M PO_4 (pH 7.4). The protein eluted from the column was concentrated by precipitation with solid $(NH_4)_2SO_4$ (added to a final concentration of 2.0 M) and dialyzed against 0.15 M NaCl–0.01 M phosphate. Any precipitate which formed during dialysis was discarded. The yield of purified antibody ranged from 35 to 65% depending on the antiserum. Judging from precipitin and supernatant analysis using DNS–HSA and DNS–BγG as precipitating antigens antibody preparations were at least 85–90% pure. Precipitation was carried out at an antibody concentration of 0.5 mg/ml.

The fluorescence exhibited by the purified antibody (activation at 316 mμ and emission at 520 mμ) indicated that as many as 5% of the combining sites of the purified antibody might be occupied by DNS-sulfonate. Purification in the presence of ^{125}I-labeled DNS–HSA indicated that essentially none of the original precipitating antigen was retained in preparations of purified antibody. Concentrations of purified anti-DNS antibodies and rabbit γ-globulin were determined by absorbance at 280 mμ (E_{280} 1 mg/ml per 1 cm, light path = 1.5). This figure was an average value, obtained by direct determinations on three anti-DNS antibodies by correlating ultraviolet absorbance and protein dry weight.

Method II. Rabbit anti-DNS antibody also was purified using a solid immunoadsorbent, a covalent conjugate of DNS–human serum albumin with bromoacetylcellulose (Robbins *et al.*, 1967). The relative amounts of resin and antibody used are given in Table II. Absorbed antibody was eluted from the resin with DNS-sulfonate, essentially as described in method I, following which the antibody–hapten solution was

per mole of antibody; Q_{max} is the maximal percentage of quenching of purified antibody fluorescence in fluorescence quenching titrations. Association constants (in liters mole^{-1}) are abbreviated M^{-1}; DNS_{18}-BγG, a DNS-bovine γ-globulin conjugate with an average of 18 DNS groups/molecule of protein.

passed through Dowex 1-X8. Antibody precipitability was in the range of 80–85%.

All binding studies were done in 0.15 M NaCl–0.01 M phosphate (phosphate–saline) unless otherwise specified. Fluorescence measurements were obtained using an Aminco-Bowman spectrofluorometer as described in the companion paper (Parker *et al.*, 1967). The activation and emission wavelengths for routine titrations were 340 and 480 mμ, respectively.[2] Exactly 1.0 ml of a solution of γ-globulin in 0.15 M NaCl–0.01 M phosphate was added to a clean dry cuvet. Titrations were carried out with whole globulin fractions or purified antibody. The total protein concentration was varied over a wide range but if the 280-mμ absorbance of the solution was greater than 4.5 (corresponding to an absorbancy at 340 mμ of about 0.060) high protein fluorescence blanks were obtained. The proportion of the total globulin fraction which was anti-DNS antibody varied widely and a preliminary titration was necessary to establish optimal titration conditions. Once the approximate concentration of anti-DNS antibody had been established from an earlier fluorometric titration or some independent assay for antibody, titrations were usually performed at an antibody concentration of 30 μg/ml. When antibody of especially high affinity ($>3 \times 10^7$ M^{-1}) was anticipated titrations were carried out at concentrations as low as 4 μg/ml. Routine titrations were performed at 30° in a jacketed cell holder maintained at constant temperature by a circulating water bath. A standard solution of ε-DNS-lysine in 0.15 M NaCl–0.01 M PO_4 was read intermittently during the titration. A dilute solution of ε-DNS-lysine (varying in different titrations from 0.5 to 6.0 mμmoles/ml in phosphate–saline) was added to the antibody solution in 0.01–0.03-ml increments until the total solution volume was 1.2 ml. It was often advantageous to use several solutions with different concentrations of ε-DNS-lysine in this portion of the titration curve in order to cover a maximal range in values of r (the ratio of moles of hapten bound to moles of antibody). This was especially desirable in titrations involving antibody of relatively low affinity. After the addition of the initial 0.2 ml of ε-DNS-lysine two additions of 0.04 ml of a 4×10^{-4} M solution of ε-DNS-lysine were made completing the titration. Using this procedure the ratio of the final ligand concentration to the concentration of antibody sites was 80 or greater.

In interpreting the binding data it was necessary to know the relative fluorescence of free and bound ε-DNS-lysine at 480 mμ (activation at 340 mμ). These parameters were designated as Ff (340–480) and Fb (340–480), respectively, and expressed in terms of the number of fluorescence units given by the free or bound hapten at a concentration of 1.0 mμM/ml. It was convenient to select an arbitrary constant value for Ff (340–480) and adjust the fluorometer sensitivity setting so that this value was obtained in every titration. Fb (340–480) varied with the antibody preparation. For most preparations of anti-DNS antibody Fb/Ff (340–480), the ratio to bound to free hapten fluorescence at 480 mμ was in the range of 150–200 to 1. Fb (340–480) was determined in the preliminary titration of antibody employing a relatively concentrated solution of globulin (usually a 1:10 or 1:20 dilution, corresponding to an antibody concentration of 100–400 μg/ml). Under such conditions, unless antibody affinity was unusually low ($<1 \times 10^6$ M^{-1}) there was a linear increase in fluorescence in the early part of the titration curve. The initial slope could then be used to determine Fb (340–480). This corresponded to the region of the binding curve in which the number of antibody binding sites was high relative to the total hapten concentration and essentially all of the added hapten was bound. The validity of this determination is discussed in more detail in the Results section. Another way to determine Fb (340–480) was to relate fluorometric data to data obtained by equilibrium dialysis. The relatively close correspondence between stoichiometry and association constants obtained by the two methods (see Table I) indicated that heterogeneity of Fb ordinarily was not of a magnitude which precluded obtaining accurate binding data by fluorescence enhancement.

The actual calculations were made *via* a computer program devised by Mr. George Whitlow of the Computer Center at Washington University School of Medicine (under Grant NIH FR00215 and NSF G22296). Sample calculations have been presented in tabular form elsewhere (Parker, 1967). The calculation is based on the principles previously outlined by Klotz (1947). Average antibody affinity was calculated by means of the Sips equation as described by Nisonoff and Pressman (1958) and by Eisen and Siskind (1964a,b). On the basis of triplicate titrations standard deviations for individual points on the curve usually were of the order of 0.02–0.03. The amount of information obtained in routine titrations was analyzed as described by Weber (1965) using the function $J(p)$

$$J(p) = 4p(1 - p)$$

where p is the probability of binding. By summing up the information obtained at individual points in the titration the total information about binding could be readily calculated. In most titrations between 6 and 10 binary units of information were obtained.

Equilibrium dialysis was carried out as described by Eisen (1964b). Dialysis was performed in plastic cells, each chamber of which had a 0.8-ml capacity (available from Technilab Instruments, Los Angeles, Calif.). Tritium-labeled ε-DNS-lysine (see Parker *et al.*, 1967) (sp act. 24 mc/mmole) was the hapten employed. The antibody concentration varied from 20 to 200 μg/ml depending on antibody affinity. Six or seven concentrations of hapten usually were employed to cover a broad range of ratios of hapten molecules to antibody sites. Dialysis with mixing by gentle rotation was

[2] The emission maximum for antibody-bound dye, uncorrected for instrument factors, is usually between 480 and 486 mμ. Using corrected spectra (see Parker *et al.*, 1967) the emission maximum usually is between 496 and 503 mμ.

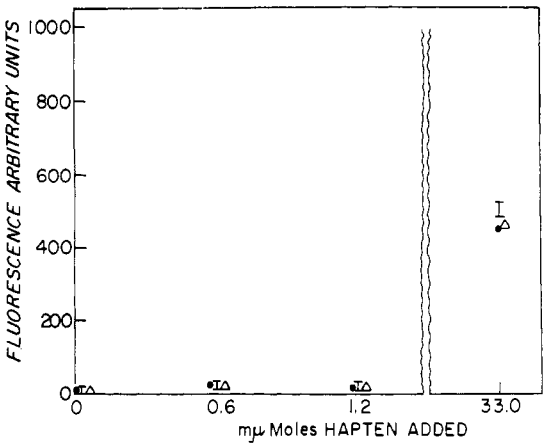

FIGURE 1: Changes in ε-DNS-lysine fluorescence in the presence of partially purified globulin. (●——●) In phosphate–saline; (△- - -△) with nonspecific γG-globulin purified by DEAE chromatography (280-mμ absorbancy, 2.0); (I) with nonspecific globulin partially purified by precipitation with 40% ammonium sulfate (280-mμ absorbancy, 2.0). (The top and bottom bars indicate the range observed with 15 different globulin fractions.) The protein and saline fluorescence blanks are subtracted; excitation 340 mμ; fluorescence 480 mμ; temperature 30°.

continued for 16 hr at ambient temperature (30° or 4°). Aliquots of both the inside and outside solutions were counted by liquid scintillation using Bray's (1960) solution as the phosphor. The counting efficiency was about 15% with the mixtures used. At the range of concentrations of ε-DNS-lysine which were employed absorption or emission by ε-DNS-lysine itself did not affect counting efficiency. With globulin fractions obtained by precipitation with ammonium sulfate small corrections were made for nonspecific binding of the hapten. These corrections were based on values obtained with globulin fractions from the same animals before immunization. The extent of nonspecific binding was similar with globulin fractions obtained from different animals.

Results

Various ways of purifying anti-DNS antisera were evaluated in an effort to maximize antibody recovery and minimize nonspecific binding of ε-DNS-lysine by nonantibody proteins. In globulin fractions obtained by two precipitations with $(NH_4)_2SO_4$ at 40% of saturation immunoelectrophoresis revealed small amounts of contaminating proteins in the α and β regions. In some instances traces of albumin also were present. Nonetheless, an adequate reduction in nonspecific binding was obtained (Figure 1) and there was little variation in the range of nonspecific binding obtained with preparations from different animals. Even less nonspecific binding was evident in preparations isolated by precipitation with 33% $(NH_4)_2SO_4$; "nonspecific" γ-globulin fractions obtained by precipitation with Na_2SO_4 or by DEAE chromatography did not alter ε-DNS-lysine fluorescence at all under the usual titration conditions. On the basis of this evaluation all preliminary screening of antisera was done with 40% $(NH_4)_2SO_4$ fractions. Subsequently highly purified γ-globulin fractions were prepared to extend the results obtained with the less pure protein fractions.

The fluorescence spectra of ε-DNS-lysine–anti-DNS

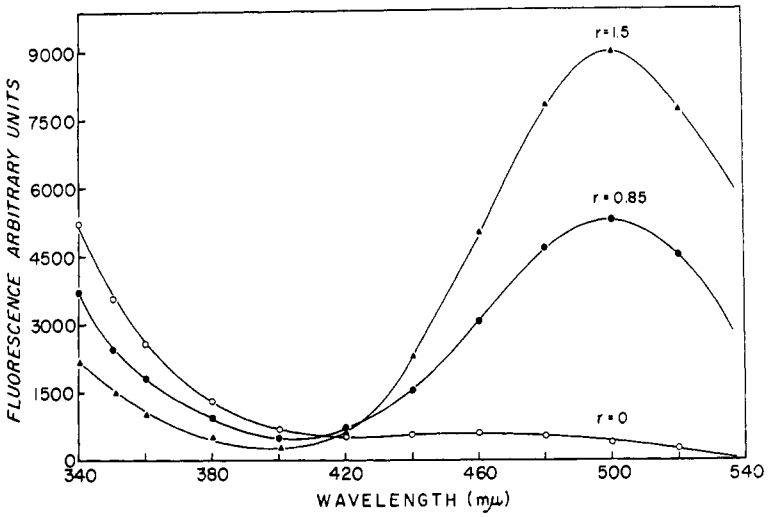

FIGURE 2: Fluorescence spectra of ε-DNS-lysine–anti-DNS antibody complexes at $r = 0$, 0.85, and 1.5. r is moles of hapten bound per mole of antibody. r was determined from independent equilibrium dialysis experiments and on the basis of the assumed value of r a small correction was made for free ligand fluorescence. The antibody was specifically purified by method I (see text). Antibody concentration was 100 μg/ml. Spectra are corrected for instrument factors; excitation 280 mμ, temperature 30°, solvent phosphate–saline.

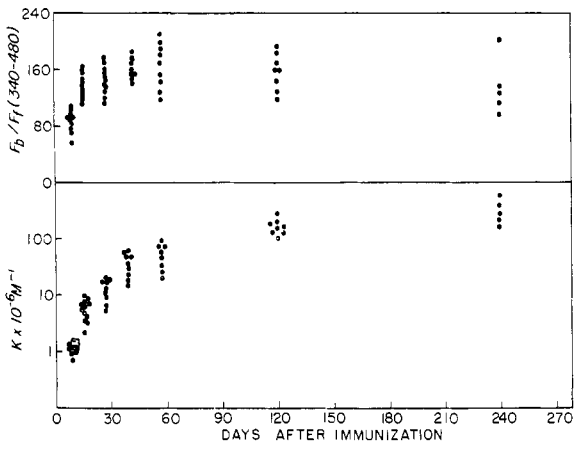

FIGURE 3: upper figure: Variation in enhancement of ε-DNS-lysine fluorescence by anti-DNS antibody (Fb/Ff (340–480), see Materials and Methods) with time after immunization. Eleven rabbits were immunized with DNS_{360}–hemocyanin. The individual antisera were partially purified by precipitation with 40% $(NH_4)_2SO_4$ before fluorometric titration. lower figure: Variation in the affinity of anti-DNS–hemocyanin antibody for ε-DNS-lysine with time after immunization. The antibody preparations are the same ones which are presented in the upper figure. The average association constant was determined by fluorometric analysis (see text). Analyses in both figures were made in phosphate–saline at 30°; excitation 340 mμ; fluorescence 480 mμ.

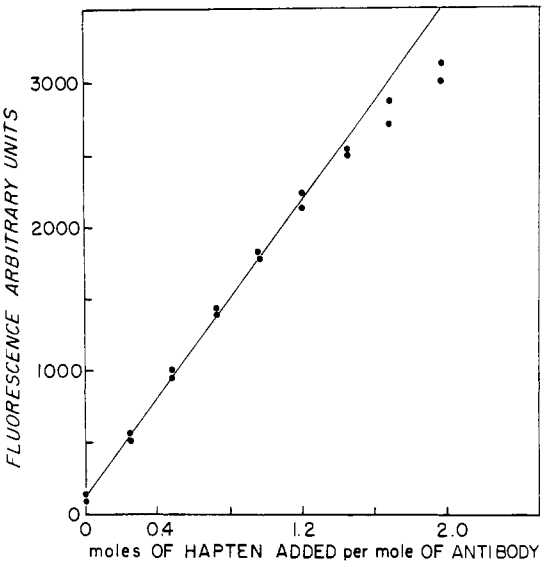

FIGURE 4: Titration of specifically purified rabbit anti-DNS antibody with ε-DNS-lysine. The antibody was at a concentration of 1.67 mg/ml. On the basis of equilibrium dialysis the antibody had an average association constant for ε-DNS-lysine of 2.4×10^7 M^{-1} at 30°. The heterogeneity index (Eisen and Siskind, 1964a,b) had a value of 0.45. The molecular weight of rabbit γ-globulin was taken to be 145,000 and the valence is taken to be 2.0. In the figure the total fluorescence is corrected for changes in volume during the titration. The titration was carried out at 30° in a 0.2-ml cell to minimize self-quenching; (light path, 3 mm). excitation 340 mμ; fluorescence 480 mμ; temperature 30°; solvent phosphate–saline.

antibody complexes, excited at 280 mμ, where both antibody and ε-DNS-lysine absorb, are shown in Figure 2. With the addition of increasing amounts of hapten there was a decrease in protein fluorescence and an increase in bound ligand fluorescence. With increasing ratios of hapten to antibody (from an r (moles of hapten bound per mole of antibody) of 0.85 to $r = 1.5$) there was a very slight shift in the emission spectrum of the bound dye to a higher wavelength. The emission maximum remained at or near 496 mμ (with a possible increase of 1 mμ) but the ratio of relative fluorescence intensities at 520 and 480 mμ rose from 0.95 to 0.96. The quantum yield (see Parker *et al.*, 1967) for the second population of antibodies (the antibody titrated from $r = 0.85$ to 1.5) was similar to that of the population titrated at $r = 0.85$ (about 0.76 and 0.80, respectively). The relationship of increases in ligand fluorescence to decreases in protein fluorescence also was examined. The point of intersection of the fluorescence curve at $r = 0$ and 0.85 was at 415 mμ. At $r = 1.5$ the intersection of the fluorescence curve with that of the curve for $r = 0$ was at 418 mμ. Thus an isoemissive point could not be demonstrated for this antibody preparation. Failure to demonstrate an isoemissive point could be due to heterogeneity of fluorescence quantum yield of the ligand (Anderson and Weber, 1965), but another possible explanation would be unequal quenching of the protein fluorescence of different antibody molecules by the ligand (McQuigan *et al.*, 1966).

In serial bleedings from individual animals immunized with DNS–hemocyanin in complete adjuvant, Fb/Ff (340–480), the ratio of bound hapten fluorescence to free hapten fluorescence measured at 480 mμ varied from as low as 33 at 12 days after immunization to as high as 220 several months after immunization (Figure 3). Relatively early in the immune response (1–4 weeks) Fb/Ff (340–480) rose approximately in proportion to increases in antibody affinity. Later in the immune response, however, Fb/Ff (340–480) actually fell in most instances while the intrinsic association constant continued to increase. Since Fb/Ff (340–480) varied between individual rabbits, the method of immunization (M. Z. Ljaljevic, unpublished data) and time after immunization each antibody preparation had to be calibrated individually with respect to its average fluorescence enhancement constant.

In view of the evidence that Fb/Ff (340–480) could vary considerably during the course of immunization the extent of Fb heterogeneity in antibody obtained by a single bleeding of an individual animal had to be examined. This question was evaluated in several

TABLE I: Correlation of Fluorescence Enhancement with Equilibrium Dialysis.

No.	Antibody Prepn[a] Method of Purifcn	Time after Immunization	Av Assocn Constant $\times 10^{-6}$ M^{-1} at 25°		Rel Antibody Concn Enhancement/ Dialysis
			Equil Dialysis	Fluorescence Enhancement	
17	40% $(NH_4)_2SO_4$	6 weeks	18.0	30.0	1.02
17	Purified AB	6 weeks	12.0	9.0	1.05
20	40% $(NH_4)_2SO_4$	6 weeks	23.0	30.0	0.92
20	Purified AB	6 weeks	24.0	20.0	0.94
Pool	40% $(NH_4)_2SO_4$	7 weeks	10.0	18.0	0.96
3	DEAE 0.02	4 weeks	5.0	6.5	1.05
8	40% $(NH_4)_2SO_4$	9 days	2.5	2.0	1.18
4	40% $(NH_4)_2SO_4$	41 days	12.0	34.0	0.90
4	40% $(NH_4)_2SO_4$	8 months	150.0	>200.0	0.80
4	DEAE 0.02	8 months	150.0	>200.0	0.92
4	DEAE 0.15	8 months	300.0	100.0	0.92
5	40% $(NH_4)_2SO_4$	27 days	5.0	7.0	1.07
5	40% $(NH_4)_2SO_4$	8 months	300.0	>200.0	0.88
5	DEAE 0.02	8 months	300.0	200.0	1.08
5	DEAE 0.15	8 months	200.0	140.0	0.88

[a] All the antibody preparations were from animals immunized with DNS–hemocyanin with the exception of 3 where immunization was with DNS_{30}–human serum albumin. Purified antibody refers to antibody isolated by specific precipitation and elution with hapten (see text); 40% $(NH_4)_2SO_4$ refers to antibody-containing globulin fractions isolated by precipitation with 40% $(NH_4)_2SO_4$; DEAE 0.02 and 0.15 refer to antibody-containing γ-globulin fractions purified by DEAE chromatography and eluted by 0.02 M phosphate (pH 7.0) and 0.15 M phosphate (pH 6.0), respectively (see Figure 5).

different ways: (1) by performing titrations under conditions in which if no heterogeneity were present a linear increase in fluorescence would be expected up to the point where at least 80–90% of the antibody sites was occupied; (2) by comparison of equilibrium dialysis and fluorescence enhancement in regard to calculated antibody concentration and affinity; (3) by titration of specifically purified anti-DNS antibody isolated by specific precipitation or by a specific immunoabsorbent; and (4) by examining antibodies which were fractionated with respect to average charge on DEAE-cellulose.

The results of a representative titration of a relatively concentrated solution of specifically purified rabbit anti-DNS–hemocyanin antibody with ϵ-DNS-lysine are shown in Figure 4. The titration was performed in a microcuvet at an antibody concentration of 1.67 mg/ml. The increase in fluorescence was essentially linear until at least 75% of the antibody sites was occupied. Deviation from linearity in the later part of the titration curve was due at least in part to nonstoichiometric binding of ligand in this region. Judging from results of equilibrium dialysis roughly 5–10% of the hapten should have been free at the last point on the curve. Thus in this titration a value for Fb/Ff (340–480) obtained early in the titration curve would be a good approximation of the average Fb/Ff (340–480) of the entire antibody population. And analogous titrations with other anti-DNS antibody preparations led to a similar conclusion.

To further validate the fluorometric titration as a quantitative measure of the antibody–hapten reaction extensive experiments were performed correlating results of fluorescence enhancement and equilibrium dialysis. Representative data are given in Table I. There was good agreement between the two techniques, both in regard to the number of antibody sites and the average association constant. The maximal deviation in terms of number of binding sites was about 20%. A discrepancy between the two assays may not always indicate an inaccuracy in the fluorometric method. In the fluorometric technique quantitative information about binding can be obtained at high ratios of hapten to antibody. In dealing with antibody of relatively low affinity sites may be titrated during fluorometric analysis which would not be detected by equilibrium dialysis unless a much higher concentration of antibody were used. This may explain the fact that in antibody preparation 8, obtained 9 days after immunization (Table I) more binding sites were obtained by fluorescence enhancement than by equilibrium dialysis. The amount of antibody determined by quantitative precipitin analysis was 5–30% lower than the amounts indicated by equilibrium dialysis and fluorometric titration.

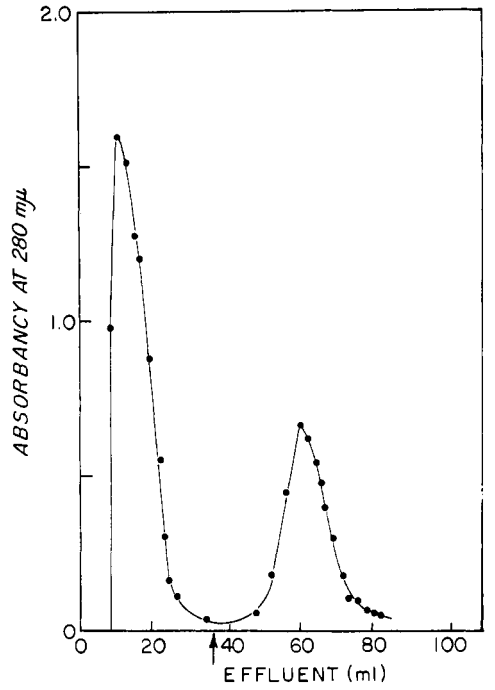

FIGURE 5: Chromatography of a rabbit globulin fraction on DEAE-cellulose. The resin and the protein were equilibrated with 0.02 M phosphate (pH 7.0). Elution from the column was initiated with this buffer. After collection of the first protein fraction was completed elution was continued with 0.15 M phosphate (pH 6.0) (indicated by arrow). The wet volume of the resin was 25 ml. The column was monitored by 280-mμ absorbance using E_{280} (1 mg/ml per 1-cm light path) 1.5 for the starting protein and both DEAE fractions. On this basis the column was loaded with 33.3 mg of protein and 20.0 mg were recovered in fraction I and 7.3 mg in fraction II; temperature 24°; flow rate 10 ml/hr.

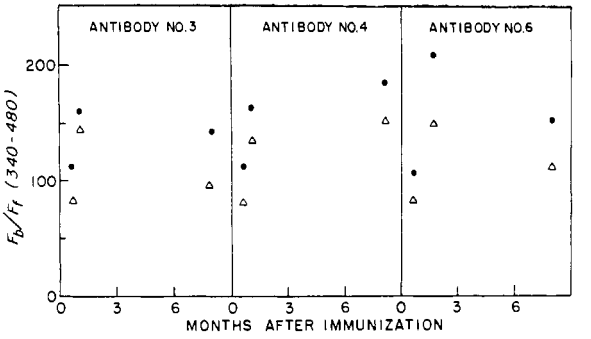

FIGURE 6: Enhancement of ε-DNS-lysine fluorescence by DEAE fractionated anti-DNS antibody. All three animals were immunized with the same amount of DNS_{360}–hemocyanin. Individual antisera obtained at the indicated times were partially purified by precipitation with 40% $(NH_4)_2SO_4$ followed by DEAE chromatography (see Figure 5). ●–●–● is DEAE fraction 0.02; △–△–△ is DEAE fraction 0.15; temperature 30°; solvent phosphate–saline; excitation 340 mμ; fluorescence 480 mμ. For a description of Fb/Ff (340–480), see footnote 1 and Material and Methods. K values for antibodies 3 and 6 are given in Table III. For antibody 4 the following values were obtained: 16 days, 0.02 fraction, $K = 4.0 \times 10^6$ M^{-1}; 16 days, 0.15 fraction, $K = 3.0 \times 10^6$ M^{-1}; 41 days, 0.02 fraction, $K = 20.0 \times 10^6$ M^{-1}; 41 days, 0.15 fraction, $K = 16.0 \times 10^6$ M^{-1}. Values for antibody 4, 8 months, are given in Table I.

Presumably, this discrepancy was due to failure to precipitate all the antibody present.

The results of fractionation of anti-DNS antibody on an immunoadsorbent are shown in Table II. Despite the fact that the antiserum had been pooled from 20 animals most of the antibody fractions were comparable with respect to fluorescence enhancement. An apparent exception was preparation 6 which exhibited a lower degree of enhancement. The validity of the fluorescence enhancement determination is less certain here because of high protein fluorescence blanks and a relatively low antibody concentration. Nonetheless, the Fb/Ff (340–480) of this fraction appeared to be significantly lower than that of the other antibody fractions.

Rabbit anti-DNS antibody also was fractionated by DEAE chromatography. A typical fractionation is shown in Figure 5. Quantitatively, 60–90% of the anti-DNS antibody and 70–90% of the total γG-globulin were eluted in the first fraction (*e.g.*, with 0.02 M phosphate, pH 7.0). The chromatographic pattern for a given globulin preparation was reproducible. On rechromatography of individual 0.02 and 0.15 M phosphate fractions essentially all the protein was eluted with the original buffer (*e.g.*, the same buffer which led to elution on the first chromatogram). In 18 of 20 instances in which antisera obtained by single bleedings of individual animals were titrated with ε-DNS-lysine, the Fb/Ff (340–480) was higher in the 0.02 M fraction. Similar results were obtained with an antiserum pool from 20 animals. The differences in Fb/Ff (340–480) between the two DEAE fractions appeared to extend throughout the period of immunization. Representative values obtained in serial titrations are given in Figure 6.

Judging from the results of immunoelectrophoresis both fractions were largely or entirely composed of rabbit γG-globulin. The γ-globulin in the 0.02 M phosphate fraction exhibited on average less electrophoretic mobility (*e.g.*, was closer to the cathode) than the 0.15 M fraction (barbital buffer, pH 8.6). However, the distribution of the two protein fractions did overlap to some extent. Several of the 0.15 M fractions did contain small amounts of β- or γA-globulin. However, the binding differences between the two fractions could not be ascribed to contamination because DEAE fractions of purified anti-DNS antibody (method II) which were free of detectable contaminating proteins exhibited similar differences.

The differences in Fb/Ff (340–480) between antibodies in the two chromatographic fractions could be

TABLE II: Fractionation of Anti-DNS Antibody with an Immunoadsorbant.

Prepn[a]	Amt of AB (mg)	Amt of Resin (mg)	% Antibody Absorbed to Resin	% Original Antibody Recovd	Fb/Ff (340–480)	Av $K \times 10^{-6}$ M^{-1}
1	20	50	36	11	160	60.0
3	20	250	60	37	180	37.0
5[b]	8.0	200	35	6	167	19.0
2		Supernatant of 1		64	181	30.0
4		Supernatant of 3		40	168	18.0
6		Supernatant of 5		26[c]	125[c]	12.0[c]
7		Original globulin fraction		100	158	20.0

[a] Preparations 1, 3, and 5 were purified anti-DNS antibodies obtained by absorption of a globulin fraction containing anti-DNS antibody with DNS–HSA–bromacetylcellulose and elution of the absorbed antibody with DNS-sulfonic acid. Preparations 2, 4, and 6 were the γ-globulin supernatants of the resin used in obtaining preparations 1, 3, and 5. They contained nonspecific γ-globulin and anti-DNS antibody which was not adsorbed by the resin. Preparation 7 is the parent globulin fraction, unexposed to resin. It was obtained from a pool of 20 antisera from animals immunized 5 weeks previously with DNS–hemocyanin. [b] Preparation 4, already absorbed with 250 mg of resin, was absorbed with an additional 200 mg of resin and the purified antibody was isolated in the usual manner. The resin was exposed to 8.0 mg of antibody of which 35% or 2.8 mg was adsorbed to the resin and 1.2 mg of antibody was recovered after elution with hapten. [c] This value is subject to some uncertainty because of relatively low antibody concentration and high protein fluorescence blanks.

correlated with differences in the emission maxima (Table III). When the dye was bound to antibodies which produced high Fb/Ff (340–480) values emission maxima generally were in the 496–500-mμ region and there was a relatively high fluorescence quantum yield. Antibodies in the 0.15 M fraction characteristically did not cause as great a shift in the emission maximum of the bound dye. Antibody 10 was a clear exception, however, in that the 0.15 M phosphate antibody fraction caused an increase in the fluorescence quantum yield of bound ϵ-DNS-lysine which was equal to the increase produced by the 0.02 M phosphate antibody fraction. The possible significance of this difference is considered in the discussion.

There were no consistent differences in the relative affinities of antibodies in the two fractions for ϵ-DNS-lysine (Tables I and III). Preliminary results have indicated that there is substantially less binding heterogeneity in the individual fractions than there is in mixtures of the two fractions. However, more studies are required before it can be concluded that this will be a consistent finding.

Discussion

The progressive alterations we observed in the affinity of rabbit anti-DNS–hemocyanin antibody for ϵ-DNS-lysine with time after immunization (Figure 3) were similar to changes exhibited by rabbit anti-DNP–bovine γ-globulin antibody (Eisen and Siskind, 1964a,b) and by rabbit anti-*p*-(*p*-iodoacetylaminobenzeneazo)-hippurate antibody (Fujio and Karush, 1966) over a similar time period. Since our observations were made with the entire γG antibody population and a number of consecutive antisera were examined they represent a significant extension over earlier observations made with specifically purified antibodies. It is now quite clear that the over-all results obtained with anti-DNP antibodies were not due to selection of certain kinds of antibody molecules during purification. These observations with three different haptenic systems utilizing either purified antibody or γ-globulin fractions afford strong evidence that under the immunization conditions employed (highly conjugated proteins given subcutaneously in complete Freund's adjuvant) progressive increases in antibody affinity are a characteristic feature of the antibody response.

Judging from the results given in Table I, fluorescence enhancement provides a useful empirical method for evaluation of the antibody–hapten interaction. Fluorescence enhancement has a distinct advantage over fluorescence quenching in that specifically purified antibody is not required. Both systems require calibration of individual antibody preparations in regard to quenching or enhancement constants before calculations of antibody affinity can be made. In fluorescence quenching the maximal quenching of antibody fluorescence (Q_{max}) is determined by additions of a large excess of hapten to antibody. Since fluorescence quenching involves a resonance transfer of excitation energy from antibody tryptophan residues to a chromophoric ligand (Velick *et al.*, 1960; Parker, 1967) it is dependent on the distribution of tryptophan in the antibody molecule. McQuigan *et al.* (1966) observed

TABLE III: Variation in the Fluorescence of Antibody-Bound ε-DNS-lysine with Different Antibody Preparations.

Antibody	Time after Immunzn (days)	DEAE Fraction[a]	Quantum Yield	λ_{max} (mμ)	Av K ($\times 10^{-6}$ M^{-1})
3	16	0.02	0.50	501	5.0
3	16	0.15	0.43	508	3.0
3	41	0.02	0.71	499	30.0
3	41	0.15	0.64	504	28.0
3	240	0.02	0.66	497	>200.0
3	240	0.15	0.51	508	200.0
6	16	0.02	0.50	501	7.0
6	16	0.15	0.42	506	5.0
6	41	0.02	0.95	499	20.0
6	41	0.15	0.71	505	9.0
6	240	0.02	0.70	498	>200.0
6	240	0.15	0.55	505	100.0
10	16	0.02	0.39	501	5.0
10	61	0.15	0.38	502	5.0
10	41	0.02	0.49	499	30.0
10	41	0.15	0.50	500	35.0
10	112	0.02	0.48	501	150.0
10	112	0.15	0.50	501	100.0
PB	35	0.02	0.70	500	20.0
PB	35	0.15	0.53	505	10.0

[a] DEAE fractions 0.02 and 0.15 refer to antibody-containing γ-globulin fractions purified by DEAE chromatography and eluted by 0.02 M phosphate (pH 7.0) and 0.15 M phosphate (pH 6.0), respectively (see Figure 5). Fractions 3, 6, and 10 are antibody-containing γ-globulin fractions from individual animals. PB is specifically purified anti-DNS antibody from a pool of 20 sera (method I). Solvent, phosphate–saline; temperature 25°. The method for determining quantum yield is described in the companion paper (Parker *et al.*, 1967).

increases in Q_{max} several months after immunization which appeared to be related primarily to increases in the average number of tryptophan residues in the Fd portion of antibody molecules.

The marked variation during immunization in the fluorescence of antibody-bound ε-DNS-lysine (Figures 3 and 6; Table III) is not explicable on the basis of alterations in tryptophan content. When activation of fluorescence is at 340 mμ not more than 2–7% of the total fluorescence exhibited by the bound dye can be due to excitation energy transfer. Thus any alterations in the fluorescence spectrum must be due to changes in the character of the antibody combining site itself. The fluorescence quantum yield of antibody-bound dye and the Fb/Ff (340–480) of the dye (see Materials and Methods) rise for the first several months of immunization during the time when antibody affinity also is rapidly increasing, raising the possibility that there may be a linear relationship between fluorescence enhancement and antibody affinity. However, studies later in the course of immunization make it clear that this is not the case. With late bleedings the quantum yield may fall quite significantly even though antibody affinity is at least as high or higher than before.

During an evaluation of the degree of binding heterogeneity in antibodies obtained from individual animals, globulin preparations were fractionated by DEAE chromatography into two populations of γG-globulin. Anti-DNS antibodies eluted from the column with 0.02 M phosphate (pH 7.0) were compared with antibodies eluted after 0.15 M phosphate (pH 6.0). In 18 of the 20 preparations examined the value for the fluorescence enhancement constant of antibody-bound ε-DNS-lysine at 480 mμ (Fb/Ff (340–480)) was higher in the 0.02 M fraction. Similar observations were made with DEAE fractions of an anti-DNS antiserum pool derived from 20 animals and with specifically purified antibody from the same pool. The differences in fluorescence enhancement between the two DEAE fractions appeared to extend throughout the period of immunization (Figure 6) and were too large to be explained by variations in excitation energy transfer. In fact anti-DNS antibodies in the 0.15 M fraction had a higher degree of tryptophan fluorescence and exhibited more extensive transfer of excitation energy from tryptophan to bound ε-DNS-lysine than antibodies in the 0.02 M fraction. The differences in Fb/Ff (340–480) were associated with differences in the average emission maxima of bound dye (Table III, antibodies 3 and 6). Within a given DEAE fraction from an individual animal, as far as could be determined, there was relatively little variation between antibody molecules in

regard to bound dye fluorescence. This conclusion was based on experiments in which the fluorescence spectra of the bound dye were compared at three or four different values of r (moles of bound hapten per mole of antibody) in the manner illustrated in Figure 2. Thus with most (but not all) anti-DNS antibody preparations the DEAE fractionation appeared to separate the bulk of the anti-DNS antibody into two rather distinct classes of γG antibody with different binding characteristics.

The fact that with most antisera differences in Fb/Ff (340–480) between the DEAE fractions were maintained throughout immunization raises several possibilities. One possibility would be that the antibody combining site is composed both of conservative and variable regions. One could assume that the antibodies in the two chromatographic fractions have somewhat different conservative regions and that this feature accounts for the lack of correspondence in the fluorescence spectra of bound ϵ-DNS-lysine between the two populations of antibody. Simultaneous changes in the character of the variable region could then be invoked to explain the progressive changes in affinity and bound dye fluorescence during immunization. The difference in Fb/Ff (340–480) would remain, however, because the conservative region continues to make a substantial contribution to the site.

The possibility that the antibody combining site may contain both a conservative and variable region has already been suggested by Singer and Doolittle (1966). They pointed out that trypsin and chymotrypsin, esterases with very different substrate specificities, contain substantial areas of homology in amino acid sequence in and near the catalytic site. Selectivity in substrate binding apparently resides in other amino acid residues adjacent to the conservative regions of these enzymes. They postulate that antibody sites also may have conservative regions with considerable similarity from antibody of one specificity to another. Taking the data presented in this paper one might assume that there are at least two major classes of γG-globulins and that these classes have somewhat different conservative regions. However, no firm conclusions are possible, in part because we have not characterized peptides from the antibody active sites. Work is in progress to develop affinity-labeling techniques for anti-DNS antibody so that this kind of analysis can be made.

In addition to the possibility that there are differences in the conservative regions of antibody sites in the two classes of γG-globulin, at least one other possible explanation for the binding differences must be seriously considered. One could assume that the anti-DNS antibody response in the two fractions involves different areas of the immunizing antigen or is to DNS–hemocyanin molecules which are substituted to different extents by hapten. In this event the binding differences would be a reflection of antigenic heterogeneity. Justification for the premise that the protein moiety can influence the distribution of rabbit γG antibody with respect to charge can be obtained from the experimental results of Sela and Mozes (1966). They separated rabbit γG antibody into two chromatographic fractions, using DEAE Sephadex, and found that the distribution of antibodies varied with the over-all charge on the immunizing antigen. Acidic protein antigens stimulated an immune response in which most or all of the antibodies were in the more positively charged γ-globulin fraction (that eluted first from DEAE Sephadex). Antibodies to basic proteins were predominantly in the more acidic γ-globulin fraction. In the immune response to uncharged haptens the over-all charge on the protein carrier governed the distribution of the antibody response. In the present work the immunizing antigen was DNS, an uncharged hapten at neutral pH and the protein carrier was hemocyanin, an acidic protein when highly substituted with DNS groups. Most of the anti-DNS antibodies (60–90%) induced by this antigen were in the more positively charged DEAE fraction. Work is in progress to determine whether or not immunization with DNS coupled to a more basic protein will give a different distribution in terms of antibody concentration and bound dye fluorescence in the two antibody populations. If a difference in charge on the immunizing antigen alters the types of antibody sites found in the two DEAE fractions, it will be necessary to discard the hypothesis that different conservative regions are involved.

Perhaps the most intriguing application of fluorescence enhancement would be to study the genetic control of antibody specificity. The effects of DEAE fractions of antibody 10 on the fluorescence quantum yield and emission of ϵ-DNS-lysine were clearly different from those of antibodies 3 and 6, even though the three animals were immunized in the same way. It seems quite possible that genetic factors are involved in these differences but supporting experimental data are not yet available. If such evidence can be obtained immune systems which utilize fluorescence enhancement would provide an attractive approach to the study of the genetics of the immune response.

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Contribution of Aromatic Residue Interactions to the Stability of Myoglobin. II. Enhancement by Aromatic Compounds of the Rate of Urea Denaturation*

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ABSTRACT: As in the case of the zinc–myoglobin reaction, benzene and other aromatics have a strong and specific enhancing effect upon the rate of urea denaturation of this protein.

Aromatic compounds exert their action on a very small portion of the myoglobin molecule, and one of the two sites with which they complex exhibits certain restraints. Both charge-transfer and hydrophobic forces are implicated in complex formation, which is believed to disrupt π-bonding interactions between the two aromatic rings of the phenylalanine residues, CD1 and 15H, and the heme.

Reaction of zinc ions with sperm whale myoglobin causes major changes in the physical and chemical properties of the protein, the most characteristic spectral change being a marked reduction in Soret-band intensity. Studies made in this laboratory (Cann, 1963, 1964a,b) have led to the conclusion that the rate-controlling step in suppression of the Soret band involves macromolecular conformational changes concomitant with rupture of the otherwise inaccessible Fe^{3+}–F8 imidazole linkage and occupancy of the imidazole group by zinc. Thus, attention is focused upon the structural complex involving the heme and adjacent portions of the protein moiety as the critical site of attack by zinc. Kendrews' 2-A model of myoglobin (Kendrew *et al.*, 1961; Kendrew, 1961, 1962; Stryer *et al.*, 1964) reveals the heme resting snugly in a hydrophobic pocket in the protein with two aromatic rings (phenylalanine residues, CD1 and 15H)[1] arranged parallel to its pyrrole rings or vinyl groups. Kendrew points out that π-bonding interactions must be significant here. That being the case, reaction of myoglobin with zinc would almost certainly necessitate rupture of these π bonds. In that event, the addition of aromatic hydrocarbons such as benzene or naphthalene to the reaction mixture should increase the rate of reaction. Such compounds might be expected to relieve the intramolecular π-bonding interactions, thereby decreasing the activation energy. Previously reported experiments designed to test these ideas (Cann, 1965) demonstrated that low concentrations of benzene and other aromatic compounds have an enormous and specific enhancing effect upon the rate of reaction. Enhancement of the rate is reversible; phenomenologically it reflects a decrease in activation energy, and mechanistically, evidently involves formation of electron donor–

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[1] In accordance with the slightly altered nomenclature to be introduced shortly by Kendrew and his co-workers (private communication from Dr. John C. Kendrew), 15H is the new designation for the phenylalanine residue formerly called H14.