Differential accessibility of liganded, high affinity rabbit anti-fluorescyl IgG antibody combining sites to the aqueous milieu has been investigated by solvent perturbation of the extrinsic fluorescence of bound fluorophore. Iodide, a dynamic quencher of fluorescein, was selected for use in these studies after examination of a number of water-soluble fluorescence quenchers. Quenching of antibody-bound fluorophore by iodide was measured with a number of liganded anti-fluorescyl IgG preparations, demonstrating partial solvent exposure of the fluorophore as well as heterogeneity of the high affinity antibody populations. Fluorescence quenching, lifetime, and absorption spectroscopy provided evidence that the antibody-bound fluorophore quenched by iodide interacted with it directly and that anomalous binding of the anion to the surface of the protein, resulting in ground state perturbations of the immunoglobulin, could not explain the observed results.

Although detailed structural features of homogeneous hapten binding myeloma proteins have been provided by x-ray analysis (1–3), and more recently by magnetic resonance techniques (4), similar data have been largely unobtainable from antigen-induced antibodies due to the problems of heterogeneity. It is, however, this property of the immune response, coupled with the question of antibody specificity, that remains one of the fundamental questions of molecular immunology. An appropriate model system to study these parameters should include one in which time-dependent maturation of the response occurs (5), resulting in the production of antibodies which possess high affinity binding sites to the eliciting antigen. Suitability of the fluorescein hapten system for studies of this nature has been described by our laboratory, as well as by others (6–9). In an attempt to understand both the generation of high affinity antibody to fluorescein-protein conjugates, and the chemical interactions involved in binding of fluorophore to specific antibody, we have undertaken a study of the absorption and fluorescent properties of antibody-bound dye (10).

Binding of fluorescein to anti-fluorescyl IgG antibody is accompanied by perturbations in both the absorption and fluorescent properties of the dye. These changes include a red shift in the lowest energy absorption band (7), induced chirality in some of the absorption bands (8), and a marked reduction in the fluorescence quantum yield relative to that of the free fluorophore (10–12). To further probe the microenvironment of the anti-fluorescyl active site, we recently studied the solvent effect of deuterium oxide (D2O) on the spectroscopic properties of ligand bound to anti-fluorescyl IgG antibody (13). We now extend those studies by determining the relative solvent accessibility of the bound ligand to quenching by an exogenous quencher.

Quenching of both the intrinsic (i.e. tryptophanyl) and extrinsic fluorescence of proteins by a variety of compounds has been observed. This approach has proven useful in the study of hapten-antibody interactions (14), enzyme conformation following substrate binding (15), nanosecond fluctuations in protein structure (16), chromophore-micelle interactions (17), and relative solvent exposure of membrane proteins as a function of membrane fluidity (18).

In this report, we examine the quenching of antibody-bound fluorescein by iodide, a known dynamic quencher of the fluorophore (19). Although there are inherent limitations in the use of a highly charged quencher for purposes of defining unequivocally the degree of solvent exposure of a given fluorophore, this experimental approach can provide important information. Therefore, in cooperation with other spectroscopic parameters, we have initiated studies to develop functional criteria for distinguishing salient differences in high affinity anti-fluorescyl antibody and gain insight into the forces involved in binding of a relatively large fluorophore to a highly specific antibody.

**EXPERIMENTAL PROCEDURES**

**Materials**

**Chemicals**—The disodium salt of fluorescein (Eastman Kodak Co.) and various preparations of fluorescein, free acid (Eastman Kodak Co., J. T. Baker Chemical Co.) were commercially obtained. The chemical purity of all stock solutions was examined by thin layer chromatography on air-dried silica gel plates (No. 6061, Eastman Kodak) first developed with acetone and then with acetone/ethanol, 95:5 (20). In this solvent system, fluorescein chromatographed near the solvent front, exhibiting a Rf of 0.94. Some solutions of the dye in water contained a pink contaminant which remained at the origin of the chromatographic plate. This substance, presumably oxidized resorcinol, has been reported by other investigators as a common contaminant of fluorescein preparations (21, 22). Methods utilized to purify these preparations included anion exchange chromatography on DEAE-cellulose (22), preparative thin layer chromatography (20), and repeated (three times) acid/base recrystallization (23). The fluorescence purity of all stock solutions of fluorescein used in these studies was assayed by fluorescence emission spectra and, more

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spectrophotometrically (10). Have used both techniques and found that over the range of I- used this titration with an identical experiment with NaCl corrects for the ionization spectrum of pure solvent added to the digitally displayed spectrum integral. The step spectra in 1 nm steps with 100 samplings recorded/step, the average being calculated by passage over a Dowex 1-X8 column equilibrated in 20 mM KPO, buffer, pH 8.0, and the haptenic substitution was determined spectrophotometrically (10).

Fluorescyl Immunogens—Fluorescein (II)/porcine γ-globulin was prepared by reaction of fluorescein isothiocyanate (isomer II) with porcine γ-globulin at alkaline pH as described by Lopatin and Voss (7). After removal of free fluorescein and IgM by anion exchange chromatography on a dual resin (Dowex 1-X8)DEAE-column (27), the purity of all preparations was verified by immunoelectrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It is important to note that this protocol yields anti-fluorescein antibodies of the IgG class, some of which retain fluorescein bound to their active sites.

For example, after purification antibody preparations from rabbits 397, 399, and 405 had 25, 54, and 85% of their antigen binding sites inhibited with ligand, respectively.

Absorption Spectra—Absorption spectra of antibody-bound fluorescein were obtained using a double beam Beckman model 25 recording spectrophotometer.

Fluorescence Emission Spectra—Unless otherwise specified, all fluorescence measurements were made at 4°C. Technical fluorescence emission spectra of fluorescein and liganded anti-fluorescein antibody in 20 mM KPO, buffer, pH 8.0, and at varying salt concentrations were recorded on the ratioscopic spectrofluorometer originally described by Weber and Young (28), equipped with a biporal averaging circuit (29). Due to the narrow separation of absorption and emission bands, the excitation wavelength was 470 nm and fluorescence was monitored from 480 to 600 nm. Under conditions employed, the selection of these limits prevented passage of scattered light to the photomultiplier tube. The emission monochromator was incremented in 1 nm steps with 100 samplings recorded/step, the average being added to the digitally displayed spectrum integral. The step spectra were recorded on a Hewlett-Packard 7015A X-Y recorder. An emission spectrum of pure solvent (i.e. KPO, buffer) was subtracted from all experimental measurements to correct for background scatter.

Quenching Experiments with Potassium Iodide—Experiments utilizing iodide (I-) as a quencher of both free and antibody bound fluorescein were performed in two ways (30). One method involved maintaining the ionic strength of the sample constant by addition of NaCl. By changing the ratio of I- to NaCl, the quenching properties of iodide could be studied. Alternatively, a concentrated solution of KI was prepared and small aliquots were added to a cuvette containing a solution of fluorophore. Comparison of the results obtained from this titration with an identical experiment with NaCl confirmed the independence of both dilution of the sample and anomalous ionic strength effects. We have used both techniques and found that over the range of I- used (0 to 0.2 M) either method is satisfactory in experiments with fluorescein and anti-fluorescein antibody. The data presented in this report were obtained using the latter method. A stock solution of 1 M KI with 10 M Na2S2O3 to prevent I3- formation was prepared immediately prior to the titrations (30).

Fluorescence lifetimes of fluorescein (1 μM) and liganded anti-fluorescein antibody were determined at concentrations of quencher between 0 and 0.2 M on the cross-correlation phase fluorometer originally described by Spencer and Weber (24), and modified with updated electronics by SLM Instrument Co. Lifetimes were determined by both the phase shift of the fluorescence emission of the fluorophore with respect to the signal of a reference scattering solution excited with sinusoidally modulated light of fixed frequency and by demodulation of the photocurrent by the sample with respect to the degree of modulation by a reference scatterer (glycogen). An excitation modulation frequency of 30 MHz was selected for studies with fluorescein due to its relatively short lifetime (24). Excitation was selected to be 470 nm and emission was monitored through a Corning 3-69 glass filter. All measurements were made at 4°C and the average value of the lifetime determined by phase and modulation was reported.

RESULTS

Quenchers of Free Fluorophore—The selection of iodide for use in these studies was made after a number of commonly used quenchers were screened for their ability to quench free fluorescein in aqueous solution. It was critical that the compound(s) not only was chosen to be an efficient quencher of the fluorophore, but that it acts through a strictly dynamic mechanism. The results of these screening assays are summarized in Table I expressed in terms of Kq, the Stern-Volmer constant, obtained from the slopes of F0/F versus [Q] plots. Acrylamide and CsCl demonstrated no measurable effect on the fluorescence of fluorescein, while L-tryptophan, phenol, hydroquinone, N-methylpicolinium perchlorate, and iodide exhibited some quenching. The "apparent" values of Kq for tryptophan, phenol, hydroquinone, N-methylpicolinium perchlorate, and iodide were 6.7, 12.0, 16.0, 3.5, and 7.6 M-1, respectively. The term "apparent" is used here as the fluorescence lifetime of fluorescein as a function of quencher concentration, from which Kq can be unambiguously derived, was not measured for all the reported compounds.

Although L-tryptophan and hydroquinone were observed to be efficient quenchers of fluorescein, the large static component in the quenching of the fluorophore by these compounds (as seen by the large positive deviations from linearity in the Stern-Volmer plots) precluded their use. This observation, which we previously reported for tryptophan quenching of fluorescein (10), was not surprising for quenching by hydroquinone, as similar findings were reported by Weber (31) for...
the quenching of the xanthene dye, eosin. Additionally, oxidation of hydroquinone, as well as phenol, to yield colored quinoids was a persistent problem. N-Methylpicolinium perchlorate was not employed due to its relatively low efficiency ($K_Q = 3.5 \, \text{M}^{-1}$).

Quenching of Fluorescein and Tryptophan by Iodide—The dynamic quenching of tryptophan (30) and fluorescein (19) by iodide has been previously reported and is included here so that analogous experiments with antibody-bound fluorescein may be compared to that of the free fluorophore in solution. Fig. 1 shows results obtained from iodide quenching studies of tryptophan and fluorescein, graphed in terms of the Stern-Volmer equation (32):

$$
\frac{F_0}{F} = 1 + K_Q [Q]
$$

where $F_0$ is the fluorescence intensity when the concentration of quencher $[Q]$ is zero, $F$ is the fluorescence intensity in the presence of the quencher, and $K_Q$ is the Stern-Volmer constant. $K_Q$ is equal to $k^* \tau_0$, where $k^*$ is the second order rate constant for the interaction of quencher and fluorophore and $\tau_0$ is the fluorescence lifetime in the absence of quencher. Agreement with the Stern-Volmer law (i.e. linearity of $F_0/F$ as a function of increasing $[Q]$) is seen whenever a single mode of quenching (i.e. dynamic or static) exists.

It is apparent from Fig. 1 that the quenching of tryptophan by iodide fits the classical equation, the value of $K_Q \approx 10.6 \, \text{M}^{-1}$. This value agrees closely with that obtained by Lehrer (30). Iodide quenching of fluorescein appears to deviate from the Stern-Volmer law as shown by the slight curvature in Fig. 1, suggesting that a small static contribution to the observed quenching may be present (16). It will be seen below that the minor deviation seen for fluorescein quenching by iodide is not substantiated by fluorescence lifetime results and that, in fact, a strict dynamic mechanism exists. The apparent value of $K_Q$ from Fig. 1 is $7.55 \, \text{M}^{-1}$, corresponding to a $k^*$ of $3.8 \times 10^5 \, \text{M}^{-1} \, \text{s}^{-1}$. The lifetime ($\tau_0$) of free fluorescein is about 4 ns.

Quenching of Antibody-bound Fluorescein by Iodide—As mentioned previously, (see “Experimental Procedures”), purification by affinity chromatography of hyperimmune anti-fluorescein IgG antibody and subsequent elution with fluorescein results in a heterogeneous population of antibody molecules, some of which retain fluorescein bound specifically to their active sites (10). This population will be referred to as liganded. An approximate reduction of 90% in the quantum yield of fluorescence of fluorescein accompanies each specific binding (10–12).

The quenching of the antibody-bound fluorophore by iodide was tested with a number of liganded anti-fluorescein antibody preparations. Fig. 2 shows representative Stern-Volmer plots obtained from studies with three anti-fluorescein antibody populations (purified from rabbits 397, 399, and 405). These data demonstrate that the fluorescence of the bound ligand can be further quenched by iodide, indicating that both partial sol-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Stern-Volmer plot of the quenching of tryptophanyl and fluoresceyl fluorescence by iodide. Tryptophan (○—○), 20 \mu M KPO_4 buffer, pH 8.0, was excited at 295 nm ± 2.66 and the emission spectrum was recorded from 300 to 450 nm. Fluorescein (●—●), 1 \mu M in 50 \mu M KPO_4 buffer, pH 8.0, was excited at 470 nm ± 2.66 and the emission was monitored from 485 to 600 nm. $F_0$ is the integrated emission in the absence of iodide, as described under “Experimental Procedures.” All measurements were made at 4°C on the fluorometer described by Weber and Young (28).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Stern-Volmer plot of the quenching of antibody-bound fluorescein by iodide. Three preparations of purified anti-fluorescein antibody, containing specifically bound fluorescein, were excited at 470 nm ± 2.66 and the fluorescence emission scanned from 485 to 600 nm. △△△, purified rabbit 405 anti-fluorescein (II) antibody; ○—○, rabbit 399 anti-fluorescein (I) antibody; □—□, rabbit 397 anti-fluorescein (I) antibody. Bound fluorescein ligand was tested at an $A_{590} = 0.1$, while rabbit 405, 399, and 397 anti-fluorescein antibodies were at 130, 200, and 400 \mu g/ml, respectively. All measurements were made at 4°C on the fluorometer described by Weber and Young (28).}
\end{figure}
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**FIG. 3.** Technical fluorescence emission spectra of fluorescein bound to anti-fluorescyl (II) antibody. Purified, liganded rabbit 405 anti-fluorescyl (II) antibody was tested at ~133 μg/ml in 50 mM KPO4 buffer, pH 8.0. The optical density at 500 nm was ~0.1. A, emission spectra at three concentrations of iodide. Spectrum 1, [I⁻] = 0; vent exposure of the fluorophore and heterogeneity of the high affinity antibody population exist. This interpretation of the data is based on the correlation that the negative deviations from linearity seen in this figure, correspond to bound fluorophores which are inaccessible to quenching by iodide under the experimental conditions employed. Representative fluorescence emission spectra recorded in these studies are shown in Fig. 3, A and B, and are discussed under “Experimental Procedures.”

The data derived from a Stern-Volmer analysis of quenching can be further evaluated using the modified equation of Lehrer (30):

\[
\frac{F_0}{F} = \frac{1}{K_{Q} f_{A}} + \frac{1}{f_{A}}
\]

(2)

where \( F_0 \), \( f_A \), and \( K_Q \) have the same meaning as that stated previously, \( \Delta F \) is \( F_0 - F \), and \( f_A \) is the fraction of solvent accessible fluorophores. It is important to note that this expression is appropriate for fluorophores in a heterogeneous environment and supposes that those molecules which can be quenched by an exogenous quencher are quenched equally (i.e. \( K_Q \) constant for all accessible fluorophores).

A more general form of this equation which allows each accessible fluorophore to possess a unique \( K_Q \) is:

\[
\frac{F_0}{F} = \frac{1}{\Sigma f_i K_{Qi}} + \frac{1}{f_A} + \frac{\Sigma K_{Qi}}{\Sigma f_i K_{Qi}}
\]

(3)

where all the terms have their aforementioned meanings and the subscript \( i \) merely denotes the \( i \)th species. The ordinate intercept, \( \Sigma K_{Qi}/\Sigma f_i K_{Qi} \), is tantamount to \( 1/f_{A} \) and can be considered here to be the reciprocal of the maximum number of accessible fluorophores.

Fig. 4 is a modified Stern-Volmer plot of iodide quenching for antibody-bound fluorescein. The values of \( f_A \) determined from the ordinate intercepts are 20, 33, and 68% for preparations derived from rabbits 405, 397, and 399, respectively.

**Comparison of Fluorescence Quenching and Lifetimes** — Perrin (33) was the first to suggest that for strict collisional quenching:

\[
\frac{F_0}{F} = \frac{\tau_0}{\tau}
\]

where \( F_0 \) and \( \tau_0 \) are the fluorescence intensity and lifetime when the concentration of quencher \( [Q] \) is zero, and \( F \) and \( \tau \) are the measured fluorescence intensity and lifetime at a fixed value of \( Q \). The presence of a static component of the quenching alters this expression so that \( F_0/F > \tau_0/\tau \), as observed fluorescence is reduced but the apparent lifetime of the fluorophore is not affected (24, 34). Fig. 5 shows the relationship of \( F_0/F \) with \( \tau_0/\tau \) for: 1) iodide quenching of fluorescein, and 2) tryptophan quenching of fluorescein. The dynamic nature of iodide quenching of fluorescein is verified by this data ((\( dF_0/F \))/(\( d\tau_0/\tau \)) = 1), while tryptophan quenching of fluorescein is seen to contain a large static component. The important result that antibody-bound fluorescein is quenched by iodide through a dynamic mechanism is contained in Fig. 6. This suggests that those fluorophores quenched by iodide are interacting directly with the quencher and that anomalous binding of the anion to the surface of the immunoglobulin cannot explain this result. This conclusion is supported by the fact that I⁻ is an inefficient quencher of the intrinsic fluorescence of anti-fluorescyl IgG antibody at pH >3.5, implying that iodide may have little affinity for the surface of the protein.²

² R. M. Watt, and E. W. Voss, Jr., unpublished observations.
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FIG. 4. Modified Stern-Volmer plot of the quenching of antibody-bound fluorescein by iodide to determine the fraction of solvent-accessible fluorophores. Data of Fig. 2 replotted to fit the modified Stern-Volmer equation of Lehrer (30). ΔΔ, purified rabbit 405 anti-fluorescein (II) antibody; ○○, rabbit 399 anti-fluorescein (I) antibody; and □□, rabbit 397 anti-fluorescein (I) antibody.

FIG. 5. Comparison of Fo/F with Tµ/T at varying concentrations of quencher. The quenching of fluorescein by iodide (●●●) and the quenching of fluorescein by tryptophan (○○○) are contrasted. Fluorescein at a concentration of 1 μM in 50 mM KPO₄, pH 8.0, was titrated with either iodide or tryptophan at 4°C. Fluorescence lifetimes were determined on the cross-correlation phase fluorometer of Spencer and Weber (24). Tµ and T correspond to the average of phase and modulation measurements of the fluorescence lifetime of fluorescein in the absence and presence of the quencher.

Absorption Spectra—The quenching of antibody-bound fluorescein could be explained by postulating that the binding of iodide to the immunoglobulin is followed by a slight conformational change in the protein resulting in quenching. As the absorption shift of fluorescein bound to the antibody is quite distinct (7), spectra were taken of the liganded antibody in 0.18 M KI and 0.18 M NaCl to determine if the ground state of the bound fluorophore was affected by iodide. Fig. 7 shows that by this criterion no such ground state perturbation occurs and that, coupled with the emission spectra presented in Fig. 3, iodide quenching of bound fluorescein cannot be explained by either a conformational change in the antibody or by a change in the ionic strength of the solvent.

DISCUSSION

Based on the previous observation in our laboratory that deuterium oxide was at least partially accessible to the anti-
fluorescyl IgG active site (13, 14), the microenvironment, specifically the degree of exposure of the liganded site to the aqueous milieu, was further investigated by solvent perturbation techniques. As the ability of exogenously added compounds to quench both the intrinsic and extrinsic fluorescence of proteins has been widely used to study fluorophore solvent accessibility in many experimental systems, adaptation of this approach to investigate liganded anti-fluorescyl antibodies seemed appropriate. Several examples of the successful application of "fluorescence perturbation" spectroscopy, introduced by Lehrer (35) to study tryptophanyl residues in lysosome were listed in the introduction.

Before initiating studies with liganded anti-fluorescyl antibody, a number of commonly employed quenchers were assayed for their ability to quench free fluorescein. As the analysis of Lehrer (30) was to be used, the criteria for selection were simply that the compound be an efficient quencher of the fluorophore (i.e., possess a large $K_q$) and that it interact with fluorescein through a predominantly collisional mechanism. Thus, static quenching (i.e., those that form a ground state complex with the fluorophore that is nonfluorescent) would be of only limited use, even if they were reasonably efficient.

The results of screening assays performed to select suitable quenchers were listed in Table I. Although a number of compounds quenched the fluorophore, only iodide (and perhaps N-methylpicolinium perchlorate) satisfied the criteria. L-Tryptophan, hydroquinone, and phenol possessed effective quenching properties, but the former two were shown to interact with fluorescein through a complex mechanism. Although not rigorously proven, due to its similar chemical structure to hydroquinone, quenching of the ligand by phenol was thought to contain a significant static component. Acrylamide which is uncharged, highly soluble in water, and an effective quencher of tryptophan (36) did not quench fluorescein. Similarly, Cs+, which is a moderately efficient quencher of tryptophan (18), exhibited no effect on the fluorescence of fluorescein. Thus, iodide, which was readily obtainable and well known to be an efficient dynamic quencher of fluorescein (19) was selected for use in these studies.

Iodide quenching of both tryptophan and fluorescein ($K_q = 10.5$ and 7.6 m$^{-1}$, respectively) is presented in Fig. 1 and included here for comparison with data obtained from the quenching of antibody-bound fluorescein. The slight positive curvature seen in Fig. 1 was shown not to be a real exposition of a static mode of quenching, but rather may have represented the presence of iodide ions that were within the "sphere of action" of the fluorophore at the time of excitation (37). That is, at reasonably high concentrations of iodide, a situation can exist where the local concentration of quenchers about the fluorophore exceeds that of the bulk solution. The result of this heterogeneous distribution is that the quenchers close to the fluorophore have an enhanced ability to interact with it and "effectively" form a dark complex, thus producing anomalous quenching effects. The presence of a strict dynamic mechanism of quenching of fluorescein by iodide was verified by the equivalence of $F_0/F$ with $\tau_0/\tau$ at specified iodide concentrations (Fig. 5).

The fluorescence of antibody-bound fluorescein, although substantially quenched by the protein, was further reduced upon addition of iodide. As shown in the Stern-Volmer plots in Fig. 2, negative deviations from the classic law were observed, revealing the existence of heterogeneous populations of bound fluorophores, some that were accessible to iodide and some that were not. It is interesting to note that the three liganded anti-fluorescyl preparations tested produced different results. This point was emphasized when the data were replotted according to the modified Stern-Volmer equation of Lehrer (30) (Fig. 4). The fractions of solvent-accessible fluorophores ($f_a$), determined from the ordinate intercepts, were 20, 33, and 68% for liganded anti-fluorescyl preparations purified from rabbits 405, 307, and 390, respectively. Repetition of these experiments over approximately an 8-month period demonstrated that these values were extremely reproducible ($\pm 2\%$). As previously stated, these antibodies contained bound ligand which occupied 85, 25, and 54%, respectively, of the total antigen binding sites. Thus, it is clear, that with respect to quenching by iodide, high affinity anti-fluorescyl sites can be distinguished from each other. The apparent lack of correlation between the number of liganded sites and per cent accessibility of iodides is probably due to the heterogeneity of certain types of sites within the antibody populations studied. Although direct evidence is not presented, per cent accessibility of iodide is most likely due to the: 1) average affinity of the liganded antibody population, 2) relative distribution of affinities, 3) charge of the quencher, and 4) chemical nature of the antibody site.

Verification that the quenching of bound ligand by iodide was dynamic was presented in Fig. 6. This experiment was important as it suggested that iodide was exerting its quenching effect through direct contact with the excited fluorophore rather than binding to another site on the immunoglobulin, resulting in anomalous quenching due to ground state perturbations. This conclusion was supported by the absorption spectra shown in Fig. 7 and by the fact that the intrinsic tryptophanyl fluorescence of IgG was unaffected by iodide at neutral pH.²

Referring to Fig. 6, it appears that up to $\tau_0/\tau$ values of 1.15, $F_0/F > \tau_0/\tau$. However, at $\tau_0/\tau > 1.15$, it is seen that $\tau_0/\tau$ is now greater than $F_0/F$. Lakowicz (38) has offered an explanation for situations when $\tau_0/\tau > F_0/F$ as observed in Fig. 6. He theorized that the latter exists when there are two populations of fluorophore, both equally accessible to a quencher (i.e., $k^t$ (2)), but with different fluorescence lifetimes. The lifetime measurements plotted in Fig. 6 represent an average of both phase and modulation ($\tau_0 \approx 2 \text{ ns}$). Although phase lifetime measurements were usually slightly lower than modulation measurements, both decreased to the same degree upon quenching by I−. Since it is reasonable to assume that there are two or more subpopulations of liganded anti-fluorescyl antibody within each preparation, it is not clear whether the deviations from linearity noted in Fig. 6 ($\tau_0/\tau > F_0/F$) are due to heterogeneous lifetimes, unequal fluorophore accessibility, or both. Finally, since $F_0/F > \tau_0/\tau$ is not observed throughout the plot, the quenching interaction is believed to be dynamic as suggested by Perrin (33).

In conclusion, the data presented demonstrate that at least some species of high affinity anti-fluorescyl antibody active sites are accessible to the aqueous milieu and, moreover, it was observed that liganded anti-fluorescyl antibodies could be distinguished by the availability of the bound fluorophore to quenching by iodide. The inability of I− to quench some fluorophore ligand bound to antibodies may correlate to the data of Hsia and Little (39) which suggested that the depth of high affinity anti-hapten antibody combining sites was greater than the corresponding sites on molecules possessing lower affinity.

Acknowledgments—We would like to extend sincere thanks to Dr. Gregorio Weber and members of his laboratory for the use of their excellent fluorescence instruments.

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