Kinetics and Mechanism of Deuterium Oxide-induced Fluorescence Enhancement of Fluorescyl Ligand Bound to Specific Heterogeneous and Homogeneous Antibodies*

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Comparative kinetics studies of ligand dissociation and D₂O enhancement were performed with both heterogeneous and homogeneous anti-fluorescyl immunoglobulin G antibodies. Heterogeneous rabbit and homogeneous mouse (monoclonal) antibody preparations were purified by immunoadsorption and found to be pure IgG by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoelectrophoresis. Relatively high affinities of all liganded antibody preparations were determined by dissociation rate studies, demonstrating comparatively long lifetimes for the dissociation of bound fluorescein. In addition, rabbit anti-fluorescyl preparations were found to display marked heterogeneity of off-rates while mouse monoclonal anti-fluorescyl preparations exhibited a single off-rate indicating homogeneity. D₂O fluorescence enhancement studies showed that heterogeneous kinetics was observed with both heterogeneous and homogeneous antibody active sites. Temperature studies of ligand D₂O enhancement and dissociation rates using homogeneous anti-fluorescyl antibodies revealed similar, yet different activation energies (22.7 ± 0.8 cal and 20.2 ± 0.3 cal, respectively) for both phenomena. The studies demonstrated that the anti-fluorescein antibody active site consists of both solvent accessible and relatively inaccessible components, and that the binding of ligand involves both exchangeable hydrogen atoms and other as yet unresolved interactions. The mechanism of D₂O fluorescence enhancement is discussed in terms of its complexity involving heterogeneous rate mechanisms.

The fluorescence quantum yield of fluorescein ligand (Φ = 0.92) is reduced by 90% or more when bound to the anti-fluorescein antibody (IgG) active site at neutral pH (1). The mechanism of fluorescence quenching has been investigated and found to be complex (2).

Recent solvent-perturbation studies demonstrated that when liganded anti-fluorescyl IgG antibody populations were equilibrated in high concentrations of deuterium oxide (D₂O), partial restoration (enhancement) of the quenched ligand's fluorescence resulted (1, 3). Fluorescence enhancement of antibody-bound ligand in D₂O was temperature- and pH-dependent (3). Low temperature (−4 °C) studies revealed complex, heterogeneous fluorescence enhancement kinetics with purified heterogeneous high affinity rabbit anti-fluorescyl IgG antibody populations. Original observations raised the question as to whether such kinetics was due to heterogeneity of antibody active sites within a population or heterogeneous mechanisms within the antibody active site.

To better understand kinetics and mechanisms, deuterium oxide enhancement of fluorescence of bound fluorescein was studied in detail with both purified heterogeneous and homogeneous (monoclonal) anti-fluorescyl IgG antibody populations. The results are discussed in terms of the rates of solvent exchange and the influence of bound ligand upon observed kinetics. Antibody-focused solvent perturbation studies coupled with a sensitive fluorophore-ligand system, provides a suitable experimental model to study varied effects of antigen-antibody interactions.

EXPERIMENTAL PROCEDURES

Materials
Chemicals—The disodium salt of fluorescein was obtained from Eastman Kodak Co. Chemical purity was examined by thin layer chromatography as previously described (4). Deuterium oxide (99.8 atom %) was obtained from Aldrich Chemical Co. Fluorescein (isomer I)-keyhole limpet hemocyanin was synthesized by reacting fluorescein isothiocyanate (1) with keyhole limpet hemocyanin according to the method of Voss et al. (1). Fluorescein-conjugated proteins were purified and characterized as previously described (1). Routinely, 200 to 300 fluorescein groups were substituted per keyhole limpet hemocyanin molecule (80 x 10⁻⁶ daltons).

Preparation of Immunoadsorbent and Purification of Anti-fluorescyl IgG—Immunoadsorbents were synthesized as previously described (5, 6). Anti-fluorescyl IgG antibodies were purified from hyperimmune rabbit anti-sera or mouse ascites fluid (1). Purification involved sodium dextran sulfate precipitation of lipoproteins, 50% saturated ammonium sulfate precipitation of γ-globulins and subsequent immunoadsorption (fluorescein-Sepharose 4B). Anti-fluorescein-specific antibodies were eluted (−48 h) with 0.1 M fluorescein disodium salt and dialyzed against 50 mm phosphate buffer, pH 8.0. Preparations were subjected to anion exchange chromatography on a Dowex 1-X8 (Bio-Rad) column to remove free fluorescein dianion. Eluted fractions were monitored for absorbance at 278 nm and 500 nm, and the per cent of sites containing bound fluorescein was calculated from the relative absorbances (1).

Methods
Immunization of Rabbits—Adult albino rabbits (~4 kg) were given primary and secondary immunizations (spaced 6 weeks apart) with 4 mg of fluorescein-keyhole limpet hemocyanin emulsified in complete Freund’s adjuvant (Difco). Bleedings were begun 15 days post-secondary immunization. Rabbits were bled repeatedly and reimmunized over a 1-year period.

Production of Murine Monoclonal Anti-fluorescyl Antibodies—Adult BALB/c mice were injected intraperitoneally with 0.2 ml of fluorescein-keyhole limpet hemocyanin (200 μg) emulsified in complete Freund’s adjuvant. Anti-fluorescein-secreting hybridomas were
produced by fusion of spleen cells harvested 4 days after secondary immunization with the 8-azaguanine-resistant Sp 2/0-Ag 14 myeloma cell line (7). Approximately 10^6 washed spleen cells from an immunized donor were fused with 10^3 myeloma cells using 50% polyethylene glycol 1500 as described by Galfre et al. (8). Cells were distributed into 48 culture wells (Costar 24 well cluster plates) and grown in hypoxanthine/aminopterin/thymidine selective medium. After 21 days, cells were grown in hypoxanthine/thymidine medium and culture supernatants were assayed 1 week later for anti-fluorescein activity.

Anti-fluorescein-secreting hybridomas were detected by both a radioimmunoassay and a fluorescence-quenching assay using supernatants from each well. Four hundred µl of culture supernatant, 100 µl of rabbit anti-mouse immunoglobulin, 50 µl of 1.0 m phosphate buffer, pH 8.0, and 10 µl of 3H-fluorescein-bovine serum albumin were incubated at 37°C for 1 h, 4°C for 6 to 12 h, centrifuged, and the amount of 3H-fluorescein-bovine serum albumin in the precipitate was determined in a γ counter. The fluorescence-quenching assay made use of the fact that fluorescein is efficiently quenched when bound to anti-fluorescein antibody (6). In this assay, 500 µl of supernatant was added to 500 µl of 0.2 µM fluorescein disodium salt in 100 mM phosphate buffer, pH 8.0. The reduction in fluorescence observed when anti-fluorescein antibodies are present was used as a method of screening for anti-fluorescein antibody positive wells.

Cells from anti-fluorescein positive wells were cloned on soft agar (0.2% SeaKem) using 3T8 mouse fibroblasts as a feeder layer (9). After 7 to 10 days, clones were transferred to new culture plates, grown to confluence and analyzed for anti-fluorescein activity. Clones exhibiting activity were subcultured and approximately five 10^6 anti-fluorescein-secreting hybridoma cells were injected intraperitoneally into pristane primed adult BALB/c mice to induce ascites fluid. Clones were designated by a three-number system, indicating fusion, well, and specific clone, respectively (i.e. 4-4-20 refers to the 20th clone isolated from well number 4 of the 4th fusion).

Monoclonal anti-fluorescein antibodies were purified from ascites fluid by immunoabsorption, as described above. Purity and homogeneity of the anti-fluorescein IgG antibody preparations was established by immunoelectrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, isoelectric focusing, and fluorescein ligand-binding studies. Data characterizing several anti-fluorescein hybridomas will be presented elsewhere.

Purity and Characterization of Antibody Preparations—Immu-
noelectrophoresis was performed using either rabbit anti-mouse γ-globulin or mouse anti-rabbit γ-globulin as the developing agent in a system described by Watt and Voss (10). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using 12% gels in the discontinuous-SDS buffer system of Laemmli (11) as described by Watt and Voss (10). Analytical isoelectric focusing was performed using a modified procedure of Briles and Davie (12).

**D0 Enhancement Studies**—The fluorescence of antibody-bound fluorescein in D_2O and H_2O was monitored with the time using the following protocol. Fifty µl of antibody (3.12 µM heterogeneous rabbit anti-fluorescein, 4.07 µM homogeneous mouse anti-fluorescein 4-4-20) containing bound fluorescein ligand (2.43 µM with homogeneous antibodies) was added to a cuvette and cooled to the appropriate temperature. Either 1 ml of D_2O (99.8% D_2O sealed under inert gas, Aldrich Chemical Co.) or 1 ml of H_2O (glass-distilled), containing 20 µl of 3 M Tris-HCl buffer, pH 8.0, was added to the antibody solution and fluorescence monitored in an Amino-Booan Bowman spectrophotometer equipped with a Heath recorder (model EUW-20A) and a Forma-Scientific controlled temperature bath and circulator. Samples were excited at 495 nm and fluorescence monitored through a 500-nm cut-off filter (Corning No. 3-69) at 535 nm.

In order to analyze D_2O enhancement kinetics, the following approximate rate law was derived:

\[
\frac{d(D_2O)}{dt} = k_1(D_2O)_0 - k_2(D_2O),
\]

where (D_2O) is the atom per cent composition of D_2O within the anti-fluorescein antibody active site, (D_2O)_0 is the atom per cent composition of bulk solvent, and \(k_1\) and \(k_2\) are the respective afferent and efferent rate constants of active site D_2O exchange. Since the concentration of H_2O (10−10 exchange reaction is negligible compared to the solvent D_2O concentration (~5 M), (D_2O) can be considered a constant. Integration of the rate law yields the following equation:

\[
\frac{(D_2O)}{(D_2O)_0} = \frac{k_1}{k_2} (1 - e^{-kt})
\]

Assuming that \(k_1 = k_2 = k\) (i.e. the anti-fluorescein antibody active site has equivalent solvent exchange properties for D_2O and H_2O), the equation can be expressed in the following useful form:

\[
\ln \left[ \frac{(D_2O) - (D_2O)_0}{(D_2O)_0} \right] = -kt
\]

Finally, converting to fluorescence units (FU):

\[
\ln \left[ \frac{(FU)_m - (FU)}{(FU)_m - (FU)_0} \right] = -kt
\]

where (FU)_0 is the initial fluorescence before D_2O perturbation, (FU) is fluorescence observed at time (t), and (FU)_m is the maximum fluorescence observed when D_2O exchange is complete.

All data obtained were analyzed in terms of equation 4, with initial fluorescence values obtained from antibody solutions prepared in H_2O, and maximum fluorescence values extrapolated from double reciprocal plots of (FU)^1 versus (time)^1. Data were computed and plotted using a 9821A Hewlett-Packard calculator equipped with a 9862A plotter.

Arrhenius activation energies were determined by plotting ln k versus 1/T (K). The rate constant k was computed from the least squares slope of linear data obtained from equation 4.

**Dissociation Rate Studies**—The dissociation rates of ligand bound to anti-fluorescein antibody preparations were determined using the following protocol. Fifty µl of antibody (as described for D_2O enhancement) was added to a cuvette at the appropriate temperature. One ml of 5-aminofluorescein (1 µM) in 50 mM phosphate buffer, pH 8.0, was added and fluorescence was monitored as described above. Since 5-aminofluorescein has a comparatively low quantum yield (0.003), yet binds anti-fluorescein antibodies with the same affinity as fluorescein (13), one can observe an increase in fluorescence due to the dissociation of previously quenched, bound fluorescein. Fluorescein dissociation data were analyzed and plotted by first order kinetics (equation 4). Arrhenius activation energies were determined as described above.

**Statistical Methods**—Linear data (dissociation plots, linear portion of D_2O enhancement plots, and Arrhenius plots) were regressed by least squares to obtain values for slope, x intercept, and y intercept. Standard error of slope and intercept values were determined as described by Snedecor and Cochran (14). Significance of the observed differences in Arrhenius activation energies, calculated from fluorescein dissociation and D_2O enhancement experiments, was quantitated by t statistics. T values were calculated as described by Snedecor and Cochran (14) and significance levels computed with a HP-67 programmable calculator.

**RESULTS**

Purity and Characterization of Anti-fluorescein Prepara-
tions—Heterogeneous rabbit anti-fluorescein and homogene-
ous (monoclonal) murine anti-fluorescein antibody preparations exhibited characteristic γ (IgG) mobility by immuno-
electrophoresis. SDS-polyacrylamide gel electrophoresis of reduced and alkylated samples demonstrated a 58,000-dalton component (γ heavy chain) and a 22,000-dalton component (light chain).

Isoelectric focusing of rabbit anti-fluorescein antibodies showed heterogeneous profiles (spectrotypes) exhibiting a range of pI values from 6.0 to 8.0. Monoclonal murine anti-
fluorescein antibodies (4-4-20 and 4-6-10) were found to have highly restricted spectrotypes characteristic of homogeneous preparations (15).

**Binding Characteristics of Heterogeneous and Homoge-
neous Anti-fluorescein Antibodies**—Table 1 shows the com-
parative ligand-binding properties of monoclonal murine anti-
tbody preparations 4-4-20 and 4-6-10 relative to three previ-
ously examined heterogeneous rabbit anti-fluorescein IgG prepara-
tions. Measurement of the fluorescence of bound fluorescein for preparations 4-4-20 and 4-6-10 showed a reduction of
under "Experimental Procedures." fluorescein in the presence of excess 5-mi of fluorescein as described. Measurements of fluorescence and absorption of fluorescein within these sites determined through exchange chromatography. The following binding properties were heterogeneous (i.e. nonlinear) rate kinetics (Fig. 1). Heterogeneous rabbit anti-fluorescein antibody preparation binding properties were shown a greater is bound (i.e. higher affinity sites tend to demonstrate greater concentration of bound ligand, although this phenomenon is due to the greater fraction of bound ligand when compared to lower affinity preparations. Heterogeneous anti-fluorescein antibody exhibited heterogeneous D2O enhancement kinetics, at all times and temperatures assayed. For a given preparation, D2O enhancement kinetics was always faster than ligand dissociation kinetics (see below).

Heterogeneous anti-fluorescein antibodies were purified as described under "Experimental Procedures." Higher affinity sites retain bound fluorescein after anion exchange chromatography. The following binding properties were determined through measurements of fluorescence and absorption of fluorescein within these sites.

| Antibody preparation | Per cent sites containing bound fluorescein | Per cent quenching of bound fluorescein | Per cent D2O enhancement | Per cent dissociation enhancement |
|----------------------|--------------------------------------------|----------------------------------------|---------------------------|-------------------------------|
| Heterogeneous rabbit anti-fluorescein | 397 | 25 | 90 | 39 | N.D. |
| | 399 | 54 | 90 | 56 | N.D. |
| | 405 | 85 | 90 | 79 | N.D. |
| | C12L | 40 | 95 | 47 | 240 |
| Homogeneous murine anti-fluorescein | 4-4-20 | 38 | 95 | 130 | 543 |
| | 4-6-10 | 11 | 99 | 10 | 84 |

*Calculated from the relative absorptions at 500 nm (bound fluorescein) and 278 nm (antibody).
*Per cent decrease in fluorescence of bound fluorescein relative to free fluorescein at the same concentration.
*Per cent increase in fluorescence of bound fluorescein in D2O relative to H2O.
*Per cent increase in fluorescence after dissociation of bound fluorescein in the presence of excess 5-amino fluorescein as described under "Experimental Procedures."
*Values for rabbit anti-fluorescein preparations 397, 399, and 405 are from Voss et al. (1).
*N.D., not determined.

94.3% and 98.8%, respectively, compared to unbound dye. Both preparations demonstrated D2O fluorescence enhancement of bound ligand, although 4-4-20 was significantly greater (130% compared to 10% for 4-6-10). Based on these results, hybridoma 4-4-20 was selected for further studies involving D2O perturbation.

It has been observed that the degree of D2O fluorescence enhancement is dependent on D2O concentration, and the affinity of the antibody for the fluoresceyl ligand. A D2O concentration of >90% results in the maximum enhancement achievable. Experiments described in this report were performed at a standard final D2O concentration of 93.5%.

The fluorescence enhancement observed upon dissociation of bound ligand is shown in Table I. The maximum enhancement attained can be expected to be a function of the degree of quenching of bound ligand and the fraction of ligand which is bound (i.e. higher affinity sites tend to demonstrate greater fluorescence enhancement in the dissociation experiment). Similarly, higher affinity antibody preparations have generally shown a greater D2O enhancement effect. It is possible that this phenomenon is due to the greater fraction of bound ligand which would be present at equilibrium with such antibodies when compared to lower affinity preparations.

Kinetics of Fluorescence Enhancement in Deuterium Oxide—The relative fluorescence intensities of fluoresceyl ligand bound to purified heterogeneous rabbit anti-fluorescein IgG antibodies was monitored over time in D2O relative to H2O (Fig. 1). D2O fluorescence enhancement appeared to consist of heterogeneous (i.e. nonlinear) rate kinetics (Fig. 1). Since the kinetics of D2O enhancement involved more than a single rate, the question remained whether the complex kinetics was due to heterogeneity of the active sites or mechanisms within a single site. To resolve the problem, D2O fluorescence enhancement studies were performed with homogeneous antibodies (i.e. populations in which all sites are identical). D2O enhancement plots for monoclonal antibody preparation 4-4-20 (Fig. 2) exhibited complex, heterogeneous kinetics, with two distinct components observed. The initial component was heterogeneous and accounted for 34.5 ± 3.2% of the D2O enhancement effect. The second component appeared fairly homogeneous, and was regressed by least squares, yielding a correlation coefficient of >98%. Rate constants, determined from least squares slope, exhibited a pronounced temperature effect varying from 2.4 × 10⁻⁴ s⁻¹ at 4 °C to 0.35 s⁻¹ at 60 °C. For monoclonal preparation 4-4-20, average initial component kinetics was faster than ligand dissociation kinetics (see below), while the observed second component rate was <20% of the ligand dissociation rate.

Heterogeneous anti-fluorescein antibody exhibited heterogeneous D2O enhancement kinetics, at all times and temperatures assayed. For a given preparation, D2O enhancement kinetics was always faster than ligand dissociation kinetics (see below).
Dissociation Rates of Heterogeneous and Homogeneous Anti-fluorescyl Antibodies—To substantiate that fluorescence enhancement in D$_2$O (1), and to further characterize the heterogeneity and/or homogeneity of these antibody preparations, dissociation rate studies were performed. Dissociation rates were determined by monitoring the increase in fluorescence of dissociated fluorescein (Φ = 0.92) in the presence of a 10-fold excess of 5-aminofluorescein (Φ = 0.003). Rate plots of ligand dissociation for heterogeneous and homogeneous IgG preparations are also shown in Figs. 1 and 2, respectively. Heterogeneous antibodies (Fig. 1) demonstrated a nonlinear curve, indicative of multiple populations of fluorescein-specific antibodies with different off-rates. However, the obvious differences between curves of D$_2$O enhancement and ligand dissociation indicates that the D$_2$O phenomena is not due to D$_2$O-induced ligand dissociation.

Homogeneous antibodies (4-4-20, Fig. 2) exhibited a linear curve, consistent with a homogeneous dissociation rate. Thus, it was further demonstrated that the D$_2$O enhancement effect (heterogeneous) involves mechanisms distinct from ligand dissociation (homogeneous).

Fluorescence enhancement due to D$_2$O perturbation or ligand dissociation was quantitatively compared for heterogeneous and homogeneous preparations (Table I). Both antibody preparations (heterogeneous and homogeneous) exhibited significantly greater percent enhancements upon ligand dissociation than D$_2$O perturbation. Such results might be anticipated if one considers that the dissociation of bound fluorescein results in the alleviation of all quenching mechanisms, while D$_2$O enhancement is due to partial relief of quenching mechanisms.

Temperature-dependent Kinetics of D$_2$O Enhancement and Ligand Dissociation—It had been reported previously that D$_2$O enhancement was a temperature-dependent phenomenon (1). The rate of D$_2$O enhancement and ligand dissociation was monitored at different temperatures for both heterogeneous (Fig. 1) and homogeneous (Fig. 2) IgG antibody preparations. Both D$_2$O enhancement and ligand dissociation were found to be temperature-dependent phenomena. In order to quantify temperature effects, monocal antibody preparation 4-4-20 was examined at several temperatures (Table II) and Arrhenius plots of both D$_2$O enhancement and ligand dissociation were constructed (Fig. 3). Activation energies of D$_2$O enhancement and ligand dissociation were calculated to be 22.70 ± 0.86 and 20.24 ± 0.33 cal/mol for D$_2$O enhancement and ligand dissociation, respectively.

**Table II**

| Temperature °C | D$_2$O enhancement a | Fluorescein dissociation |
|---------------|----------------------|-------------------------|
| 4.4           | 4148 ± 86            | 4335 ± 2.1              |
| 8.1           | 1886 ± 33            | 3416 ± 1.8              |
| 12.0          | 1379 ± 39            | 2111 ± 1.5              |
| 15.9          | 896 ± 22             | 1188 ± 1.8              |
| 19.5          | 797 ± 22             | 998 ± 0.63              |
| 23.3          | 364 ± 10             | 497 ± 0.22              |
| 28.0          | 234 ± 11             | 28.96 ± 0.26            |
| 32.3          | 99.5 ± 2.7           | 15.84 ± 0.07            |
| 36.0          | 70.0 ± 2.0           | 11.75 ± 0.10            |
| 40.1          | 41.0 ± 1.5           | 7.350 ± 0.050           |
| 50.0          | 18.3 ± 1.1           | 2.639 ± 0.039           |
| 60.0          | 2.89 ± 0.08          | 2.193 ± 0.073           |

a Lifetimes (time required for fluorescence change to reach e$^{-1}$ of maximum value) were determined from reciprocal rate constants (τ = k$^{-1}$). All values and standard errors were determined by linear regression of first order kinetic data obtained at each temperature.

Analysis of D$_2$O enhancement kinetics revealed a fast heterogeneous component, and a slow homogeneous component (see "Results"). Reported lifetime values for D$_2$O enhancement were determined from the slow homogeneous component.

**FIG. 3.** Arrhenius plots of D$_2$O enhancement and ligand dissociation of fluorescein bound to homogeneous anti-fluorescyl antibodies. Arrhenius plots of D$_2$O enhancement (●-●) and ligand dissociation (△-△) were constructed. D$_2$O enhancement and fluorescein dissociation experiments were performed at various temperatures, as described in Figs. 1 and 2. The rate constant k was computed by plotting ln k versus 1/T and calculated to be 22.70 ± 0.86 and 20.24 ± 0.33 cal/mol for D$_2$O enhancement and ligand dissociation, respectively.
D$_2$O Fluorescence Enhancement

Heterogeneity of D$_2$O enhancement plots, obtained from monoclonal preparation 4-4-20 (Fig. 1), was more pronounced, initially, than at longer times. The initial heterogeneous enhancement exhibited faster average kinetics than that observed for ligand dissociation and accounted for ~34.5% of the total observed D$_2$O enhancement effect. Subsequent deuterium enhancement exhibited relatively homogeneous kinetics with a rate significantly less (<20%) than that observed for ligand dissociation, and accounted for the remaining 65.5% of the observed enhancement effect. Linear regression by least squares of the slow component showed nearly homogeneous kinetics (correlation coefficient 98%), but since the precision of the assay was ~1-2%, the presence of very slow components could not have been detected under these conditions.

Arrhenius activation energies were determined for both fluorescence dissociation and for the linear component of D$_2$O enhancement (Fig. 3). Activation energies of 20.2 ± 0.3 cal/mol and 22.7 ± 0.8 cal/mol were determined for fluorescence dissociation and D$_2$O enhancement, respectively. The 2.5 cal/mol difference in these values was significant to >98.5% (by $t$ statistics). However, since both activation energies were similar, the mechanism of D$_2$O enhancement (of the slow component) probably involves a dissociative event. The rate of D$_2$O enhancement of the slow component is <20% of the dissociation rate, implying that ligand dissociation is necessary, but not sufficient for D$_2$O enhancement. The additional 2.5 cal/mol required for D$_2$O enhancement probably accounts for the remaining events (such as deuterium-proton exchange) necessary for enhancement. Ligand dissociation was not required for the initial heterogeneous D$_2$O enhancement component, since the observed enhancement rate was much faster than the ligand dissociation rate.

In summary, D$_2$O enhancement kinetics, for monoclonal preparation 4-4-20, contained two macroscopic components: a relatively heterogeneous rapid component that accounted for 34.5% of the observed enhancement effect and did not require ligand dissociation, and a more homogeneous slow component for which ligand dissociation was necessary but not sufficient. It is presumed that deuterium exchange observed in these studies involves both solvent accessibility of exchangeable hydrogen atoms, as with the original work on insulin (17), and various binding mechanisms involving exchangeable protons within the active site. Therefore, the complexity of D$_2$O enhancement kinetics most likely results from the combined effects of solvent accessibility and fluoresceyl ligand binding within the active site of the antibody molecule. The rapid component (34.5% enhancement) may involve the former effect while the slower component (65.5% enhancement) may involve both effects.

Finally, it has become apparent that D$_2$O enhancement is a complex phenomenon, involving heterogeneous rate mechanisms. The effect is dependent on the internal diffusion of D$_2$O and the solvent exchange with protons involved in the fluorescein-quenching mechanisms within the active site. In addition, based on the total per cent fluorescence enhancement achievable in the dissociation experiment (~300%) and that observed with the same preparation with D$_2$O (130%), it becomes obvious that the quenching mechanisms within an active site are many and varied. It is likely that protonation of the fluoresceyl ligand within the active site is only one pathway by which the fluorescence of bound fluorescein may be quenched. In addition, to a variety of quenching, and hence binding modes within a single active site, we have now observed numerous differences in the affinities and binding mechanisms of different monoclonal preparations. We are

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1 Unpublished data.
continuing to use the method of deuterium exchange to further elucidate and compare the nature of the combining sites of these anti-fluorescyl antibody clones.

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