# KINETIC BEHAVIOR OF ANTIHAPTEN ANTIBODY OF RESTRICTED HETEROGENEITY BY STOPPED FLOW FLUORESCENCE POLARIZATION KINETICS*

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SUMMARY: Fluorescein, antifluorescein reactions have been studied by initial fluorescence polarization and intensity rate methods. Novel rapid stopped flow instrumentation (msec time range) as well as slower but more sensitive instrumentation has yielded second order rate constants over wide ranges in concentration and time. A bimolecular mechanism for the fluorescein,univalent antifluorescein system has been proposed in which two types of site interactions occur. This mechanism accounts for the fact that fluorescence polarization data consistently yield second order rate constants which are a factor of 10 higher than those obtained from fluorescence quenching data. Comparisons of kinetic behavior of free fluorescein and of fluorescein covalently attached to a normal IgG carrier reacting with Fab antifluorescein have suggested a "carrier effect" of a steric nature. The large equilibrium association constant for fluorescein, antifluorescein systems has been found to be due to extremely small values of the back reaction rate constants; i. e. $10^{-3}$ to $10^{-4}$ as great as those reported for DNP, anti DNP systems.

INTRODUCTION: In recent years, fluorescence polarization has been coupled to classical kinetic techniques in studies involving the rates of reaction for various antigen, antibody systems (1, 2, 3, 4). The fluorescence polarometer, (5), which was utilized in those kinetic studies as well as in recent thermodynamic studies of the fluorescein, antifluorescein systems (6) is extremely sensitive, but is limited to detecting events in time ranges of 10 seconds and above. We have now completed construction of a stopped flow device which monitors both fluorescence polarization and intensity changes simultaneously in the millisecond time range. A detailed description of this apparatus will be published elsewhere (7). Fast macromolecular reactions such as hapten, antihapten or enzyme, substrate reactions can now be readily studied by fluorescence polarization kinetic techniques. This particular communication deals with the utilization of this fast reaction polarometer in detailed kinetic studies of the hapten fluorescein reacting with antifluorescein by the method of initial rates.

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MATERIALS AND METHODS: Antibody to fluorescein was obtained from rabbits by intradermal injection of fluorescein labeled ovalbumin (dye/protein ratio = 0.8) in complete Freund's adjuvant at two sites. Each site received 2.2 mg of the fluorescein-ovalbumin in 0.2 ml of the final emulsion. This procedure was repeated four times every two weeks. Then bleedings were taken every week and booster immunizations of 50 μg/rabbit in complete Freund's adjuvant were given every two months. Immunoglobulin fractions of antifluorescein were isolated from pooled sera obtained over a period of several months about one year after the initial immunization by the same procedure used for purifying antiovalbumin IgG (4). Immunospecific divalent antibody was prepared by eluting antifluorescein from a fluorescein cellulose immunospecific column with 4M $NaClO_4$ (pH 7.0) (6). Univalent Fab fragments were prepared from IgG by papain digestion (8). Fluorescein was purified by ion exchange chromatography as described previously (9). Fluorescein labeled IgG was prepared by covalently linking fluorescein isothiocyanate to normal rabbit IgG followed by exhaustive dialysis and purification by ion exchange chromatography. The final dye to protein ratio was determined to be three by optical means (10). Antibody concentrations were determined by titration of antifluorescein activity directly with fluorescein as described in a separate publication (6). Kinetic measurements were made both with a newly constructed stopped flow polarometer* whose mixing and stopping times were less than 5 msec and with a direct readout polarometer, (5), whose time constant was of the order of several seconds. Reliable experiments in the millisecond time range with fluorescein concentrations as low as $10^{-9}$M could be obtained with the stopped flow instrument whereas with the slower apparatus fluorescein concentrations as low as $6 \times 10^{-11}$M could be readily studied. There has been previously one brief report (11) in which fluorescence polarization was used in conjunction with stopped flow techniques, but the results obtained were fragmentary and qualitive.

RESULTS AND DISCUSSION: A typical oscilloscope trace of stopped flow kinetic fluorescence polarization and intensity data is shown in Figure 1. As is obvious from the curves, when fluorescein combines with antifluorescein marked increases in the degree of fluorescence polarization occur while the

* For some time we have been using "polarometer" to denote an instrument for measuring the degree of polarization as contrasted to optical rotation. The stopped flow polarometer employed utilizes a dual phototube detector and provides means by which background fluorescence is blanked out. Simultaneous recordings of fluorescence polarization and intensity are obtained. Resolution of the instrument is limited only by mixing and stopping times down to concentrations of about $10^{-9}$ M fluorescein.

$[F] = 1.0 \times 10^{-7}M$; $[Fab] = 2.0 \times 10^{-7}M$

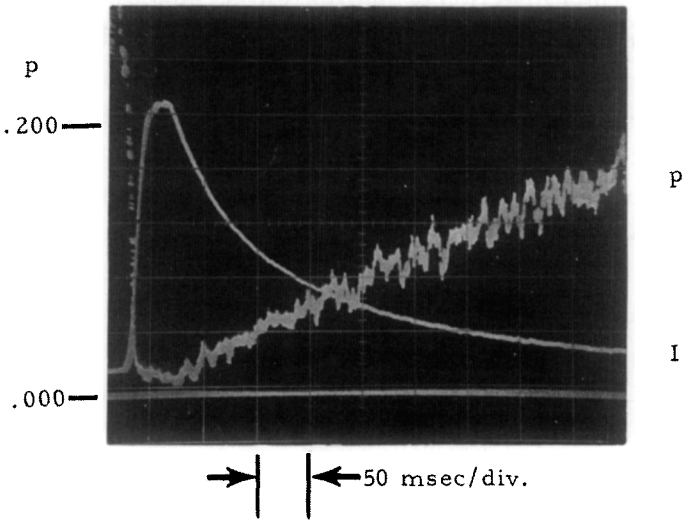


FIGURE 1: Oscilloscope trace of stopped flow fluorescence polarization and quenching kinetic curves for the fluorescein, univalent antifluorescein (Fab) reaction at 18°. The pH 7.0 buffer contained 0.15M NaCl, 0.01M $K_2HPO_4$, 0.005M $KH_2PO_4$.

fluorescence is quenched. Rate equations involving either the initial rate of change of fluorescence polarization or of fluorescence intensity have been utilized to ascertain an empirical rate law for fluorescein, antifluorescein systems. In terms of fluorescence polarization, the initial rate law can be formulated as follows (1):

$$(dp/dt)_o = k(Q_b/Q_f)(p_b - p_f)(AB)_o^{N_1}(F)_o^{N_2-1} \qquad (1)$$

where $(dp/dt)_o$ is the initial rate of fluorescence polarization change; $p_b$ and $p_f$ designate polarizations of fluorescence of the bound and free forms respectively of fluorescein (or fluorescein labeled IgG); $Q_b$ and $Q_f$ are the molar fluorescences (4) of the bound and free forms respectively of fluorescein (or fluorescein labeled IgG); $(AB)_o$ and $(F)_o$ are the initial molar concentrations of antibody and fluorescein (or fluorescein labeled IgG) respectively.

The initial rate law can also be formulated in terms of molar fluorescence as follows (3):

$$(dQ/dt)_o = k\left[(Q_b - Q_f)/M\right](AB)_o^{N_1}(F)_o^{N_2-1} \qquad (2)$$

where $(dQ/dt)_o$ is the initial rate of fluorescence intensity change and M is the total amount of fluorescent material. By plotting log $(dp/dt)_o$ or log $(dQ/dt)_o$ vs log $(AB)_o$ at constant (F) the order with respect to (AB), $N_1$, can be deter-

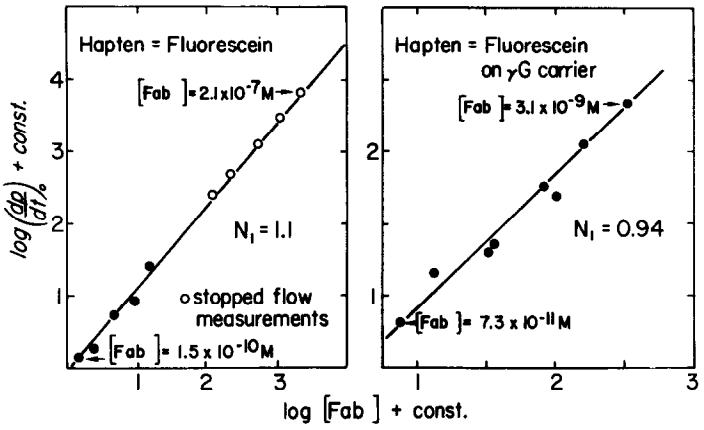


FIGURE 2: Determination of the reaction order, $N_1$, with respect to antibody concentration by the initial fluorescence polarization rate method (Equation 1) for fluorescein, univalent antifluorescein (Fab) systems; (Comparison between free hapten and hapten covalently bound to IgG carrier). All experiments were carried out in a pH 7.0 buffer solution of 0.15M NaCl, 0.01M $K_2HPO_4$, 0.005M $KH_2PO_4$. ●, White polarometer, mixing time 10 sec, (Fluorescein), $1.5 \times 10^{-10}$M; O, Stopped Flow Apparatus (Fluorescein) = $1.5 \times 10^{-8}$M.

mined. On the other hand, a plot of log $(dp/dt)_o$ or log $(dQ/dt)_o$ vs log $(F)_o$ at constant (AB) yields $N_2$, the order with respect to (F).

Examples of determinations of $N_1$ and $N_2$ by means of initial polarization rate changes are shown in Figures 2 and 3 respectively for the reaction between fluorescein and antifluorescein Fab fragments. Comparisons have been made between fluorescein covalently attached to an inert IgG carrier and free fluorescein. In both systems, a rate law which is first order with respect to antibody concentration and first order with respect to hapten or hapten-carrier concentration is followed:

$$-(d\ (\text{fluorescein})/dt)_o = k_F\ (\text{Fab})\ (\text{fluorescein}) \quad (3)$$

$$-(d\ (\text{fluorescein-IgG})/dt)_o = k_{F\text{-IgG}}\ (\text{Fab})\ (\text{fluorescein-IgG}) \quad (4)$$

In the studies of the fluorescein, antifluorescein Fab systems, experiments were carried out both with the White polarometer and the stopped flow polarometer. Utilization of both of these instruments has allowed testing of the empirical initial rate law over a factor of 1000 in Fab concentration and 300 in fluorescein concentration. The fact that both of these apparatuses yield the same second order rate constants at vastly different concentration and time ranges points up the fact, that these instruments, while measuring fluore-

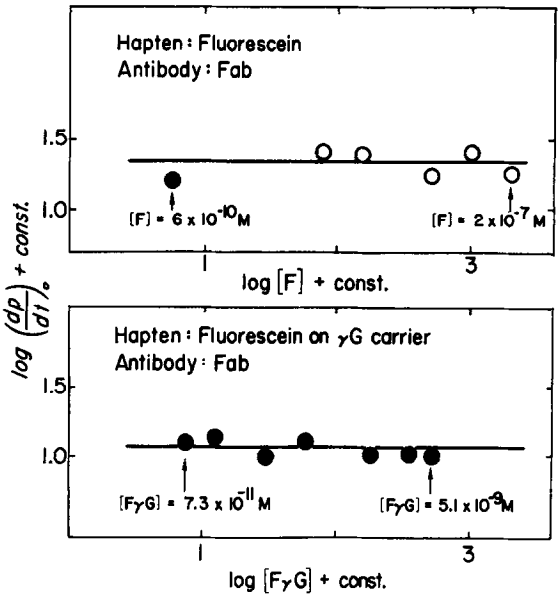


FIGURE 3: Determination of the reaction order, $N_2$, with respect to hapten by the initial rate fluorescence polarization method (Equation 2) for the fluorescein, univalent antifluorescein systems. (Comparison between free hapten and hapten covalently bound to IgG carrier). All experiments were carried out in a pH 7.0 buffer solution of 0.15M NaCl, 0.01M $K_2HPO_4$, 0.005M $KH_2PO_4$. The hapten and hapten-carrier concentrations were varied from $6.0 \times 10^{-10}$M to $2 \times 10^{-7}$M and $7.3 \times 10^{-11}$M to $5.1 \times 10^{-9}$M, respectively ●, White polarometer, (Fab) = $3.3 \times 10^{-9}$M, $(dp/dt)_o$ for this point adjusted to appropriate value at $1.1 \times 10^{-7}$M Fab according to a rate law first order with respect to (Fab); O, Stopped Flow Apparatus, (Fab) = $1.1 \times 10^{-7}$M.

scence polarization changes in a somewhat different manner, are indeed telling us a similar story. Variations of 40 and 70 fold in Fab and hapten-carrier concentrations, respectively, were made for the fluorescein-IgG, antifluorescein Fab system**.

Examples of the determination of $N_1$ and $N_2$ by means of initial rates of fluorescence intensity change are shown in Figure 4 for the fluorescein, antifluorescein Fab system. Again as in equation (3) a simple second order rate law is followed over concentration changes involving factors of 200 and 50 for Fab and fluorescein, respectively. Similar studies utilizing stopped flow

** Only the White polarometer was used in studies of the fluorescein-IgG antifluorescein Fab reaction.

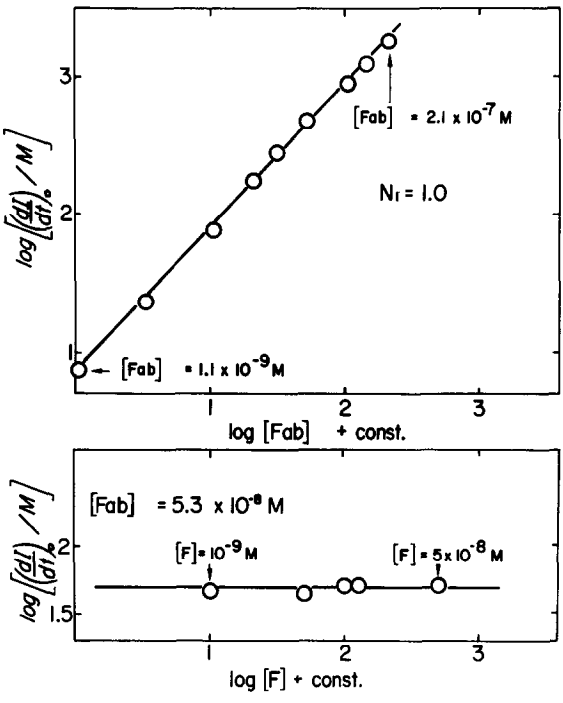


FIGURE 4: Determination of the reaction order with respect to antibody, $N_1$, and with respect to hapten, $N_2$, by the initial rate method (fluorescence quenching, Equation 2) for the fluorescein, univalent antifluorescein system. The antibody concentrations were varied from $1.1 \times 10^{-9}$M to $2.1 \times 10^{-7}$M, while hapten concentrations were varied from $10^{-9}$M to $5 \times 10^{-8}$M.

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fluorescence polarization and intensity changes have been made for immunospecifically purified divalent antifluorescein, fluorescein systems. Only the fluorescence quenching results are reported in detail as shown in Figure 5. Again a simple second order rate law is obeyed over factors of 20 and 30 in antibody and fluorescein concentrations respectively. Since the values of $Q_b$, $Q_f$, $p_b$, $p_f$ and $Q_b - Q_f$ can be determined by appropriate extrapolation techniques (12), second order rate constants have been calculated directly from equations (1) and (2) and listed in Table I. Also listed in Table I are equilibrium association constants as well as first order kinetic dissociation rate constants for several antigen, antibody and hapten, antihapten reactions studied by our group as well as those investigated by others. The average association constants, $K_o$, have been determined both by equilibrium and kinetic methods.

Several important points emerge from comparisons of the parameters listed in Table I. The second order rate constant for the fluorescein, anti-

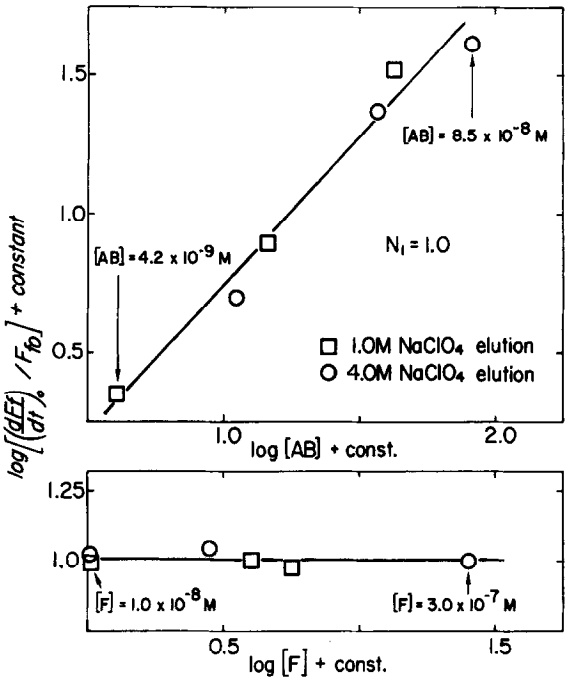


FIGURE 5: Determination of the reaction order with respect to antibody, $N_1$, and with respect to hapten, $N_2$, by the initial rate method (fluorescence quenching, Equation 2) for the fluorescein, immunospecifically purified divalent antifluorescein system. All experiments were run in 0.15M NaCl, 0.01M TRIS-HCl, 0.001M TRIS, pH 7.2 at 18°. The antibody concentrations were varied from $4.2 \times 10^{-9}$M to $8.5 \times 10^{-8}$M while fluorescein concentrations were varied from $1.0 \times 10^{-8}$M to $3.0 \times 10^{-7}$M. For the determination of $N_2$, (antibody) = $5.2 \times 10^{-8}$M. □, immunospecifically purified antibody eluted from fluorescein-cellulose with 1.0M $NaClO_4$, 0.01M TRIS-HCl, 0.001M TRIS (pH 7.2) O, immunospecifically purified antibody eluted from fluorescein-cellulose with 4.0M $NaClO_4$, 0.01M TRIS-HCl, 0.001M TRIS pH 7.2.

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fluorescein systems derived from fluorescence polarization measurements is a factor of 10 greater than the one derived from fluorescence quenching studies. Yet fluorescence polarization and fluorescence quenching equilibrium constants for the fluorescein, divalent antifluorescein system yield values of similar magnitudes (6). These facts imply that while a bimolecular combination between fluorescein and antifluorescein can be proposed, two types of site interactions may occur during formation of the hapten, antihapten complex. The more rapidly interacting sites involve both changes in the rotary brownian motion and in fluorescence intensity of fluorescein (i.e., quenching) and

Table I Thermodynamic and Rate Parameters for Hapten, Antihapten and for Antigen, Antibody Systems

| Mode of Study | System | $k_1$ ($M^{-1}$ $sec^{-1}$) | K ($M^{-1}$) | $k_{-1} = \frac{k_1}{K}$ ($sec^{-1}$) |
|---|---|---|---|---|
| Fluorescence Polarization | Fluorescein, antifluorescein (Fab) | $4.4 \pm 0.5 \times 10^8$ | $\sim 10^{11}$ | $\sim 5 \times 10^{-3}$ |
| Fluorescence Polarization | Fluorescein-IgG[a] antifluorescein (Fab) | $4.7 \pm 0.5 \times 10^7$ | ----- | ----- |
| Fluorescence Polarization | Fluorescein, antifluorescein (whole antibody, immunospecific) | $\sim 4 \times 10^8$ | $6.5 \pm 1.6 \times 10^{10}$ | $\sim 5 \times 10^{-3}$ |
| Fluorescence Quenching | " | $6.7 \pm 0.4 \times 10^7$ | $6.1 \pm 1.7 \times 10^{10}$ | $\sim 10^{-3}$ |
| Fluorescence Quenching[b] | DNP-lysine, anti DNP (divalent) | $5.0 \times 10^7$ | $4.5 \times 10^7$ | 1.1 |
| Temperature Jump[c] | Nitrophenyl, antinitrophenyl (divalent) | $1.8 \times 10^8$ | $2.4 \times 10^5$ | 760 |
| Fluorescence Polarization[d] | Fluorescein-Ovalbumin, antiovalbumin (divalent) | $4.8 \times 10^5$ | $2.4 \times 10^8$ | $2 \times 10^{-3}$ |
| Fluorescence Polarization[d] | Dansyl-BSA, anti BSA (divalent) | $3.4 \times 10^5$ | $1.7 \times 10^8$ | $2 \times 10^{-3}$ |

a The hapten to carrier ratio equals 3.2 fluorescein groups per IgG molecule. b Day, L. A., Sturtevant, J. M. and Singer, S. J., Ann. N. Y. Acad. Sci., 103, 611 (1963). c Froese, A. and Sehon, A. H., Immunochemistry, 2, 135 (1965). d Levison, S. A., Kierszenbaum, F. and Dandliker, W. B., Biochemistry, 9, 322 (1970).

relates to total changes in the degree of fluorescence polarization as the complex is formed, whereas the second type only involves quenching and excludes those fluorescein molecules which can attach themselves to antibody but do not undergo any fluorescence quenching.

The second order rate constant for the fluorescein, antifluorescein Fab system is a factor of 10 greater than the fluorescein-IgG, antifluorescein Fab system. This carrier effect seems to be of a steric rather than of a conformational nature, since both systems have similar activation energies (12).

Finally, the large equilibrium association constants for the fluorescein, antifluorescein systems are not due primarily to enormously large second order rate constants, but instead to the first order back reaction rate constant $k_{-1}$, whose magnitude is only of the order of $10^{-3}$ to $10^{-4}$ times that of the corresponding DNP, anti DNP hapten systems. This extremely slow dissociation rate constant is much more comparable to those determined for the protein antigen, antibody systems.

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