Fluorescence Polarization and Intensity Kinetic Studies of Antifluorescein Antibody Obtained at Different Stages of the Immune Response†

S. A. Levison, A. N. Hicks, A. J. Portmann, and W. B. Dandliker*

ABSTRACT: Kinetic studies of reactions between fluorescein and antifluorescein antibody produced during early, intermediate, and late stages of the immune response have been carried out utilizing both fluorescence intensity and polarizaton measurements in the static (time constant ~5 sec) and in the stopped-flow modes (time constant ~5 msec). During maturation of the immune response, it was found that the "on" second-order association rate constant increased its value only by a factor of three, whereas the "off" dissociation first-order rate constant decreased by a factor of over 1000. Hence, it is the rate of dissociation which largely determines the stability of the hapten–antihapten complex. Furthermore, since second-order rate behavior was found for even heterogeneous antibody, most of the heterogeneity with respect to binding affinity occurs as a result of the heterogeneity in the rate of dissociation of the hapten–antihapten complex and not from the primary combination of hapten and antibody. Antifluorescein antibody which exhibits both high binding affinity ($K \sim 5 \times 10^{11}$ M^{-1}) and homogeneity with respect to equilibrium binding has been shown to obey second-order association kinetics over wide ranges in concentration. Despite the fact that the value of the second-order rate constant for this fluorescein–antifluorescein reaction is as large as that for most other hapten–antihapten reactions (1.4×10^8 M^{-1} sec^{-1}), the binding reaction has an appreciable activation energy (7 kcal/mol). This is true for both divalent and univalent antibody. Furthermore, the reaction rate parameters are markedly affected by specific anions. The value of the second-order rate constant (18.5°) increases according to the following scheme: salicylate < trichloroacetate < SCN^- < ClO_4^- < Cl^- < F^- < phosphate. The activation energy increases as follows: trichloroacetate < phosphate < F^- < Cl^- < ClO_4^- < SCN^- < salicylate, whereas estimates of the entropy of activation indicate that $\Delta S^\ddagger$ increases as follows: trichloroacetate < phosphate ~ F^- < Cl^- ~ ClO_4^- < SCN^- < salicylate. The same mechanism which was previously proposed by us for the antigen–antibody reaction is also consistent with the kinetics of the fluorescein–antifluorescein reaction. This mechanism postulates a bimolecular process with structural rearrangements (conformational changes and/or the loss of water) in the formation of the transition state complex. The reaction between the fluorescein hapten and its antibody hence is not diffusion limited.

Detailed kinetic investigations of hapten–antihapten reactions have been limited to studies involving such classical determinants as phenyl arsonate (Froese et al., 1962; Kelly et al., 1971), *p*-nitrophenyl (Froese, 1968), 2,4-dinitrophenyl (Day et al., 1963; Pecht et al., 1972; Barisas et al., 1975), and 2,4,6-trinitrophenyl (Barisas et al., 1975). The hapten–antihapten reaction in these systems has been assumed to obey second-order kinetics and to be essentially diffusion controlled. However, recent kinetic studies of the binding of various nitrophenyl ligands to the MOPC-315 protein (Haselkorn et al., 1974) have revealed that this association reaction may not be diffusion controlled. The measured values of the second-order association rate constant for these systems lie in the range of 10^7–10^8 M^{-1} sec^{-1}, while the measured values of the dissociation first-order rate constant range from 1 to 10^3 sec^{-1}. Unfortunately, except for the myeloma proteins, the preparations studied were for the most part heterogeneous with respect to binding affinity.

Efforts in our laboratory have recently centered on the equilibria and kinetics of a somewhat different type of hapten–antihapten system, namely, fluorescein–antifluorescein (Portmann et al., 1971, 1975; Levison et al., 1971). The antifluorescein preparations that we have studied exhibited an extremely high binding affinity, as well as homogeneity with respect to binding. The large equilibrium association constant (~10^{11} M^{-1}) has been shown to be due to the extremely small "off" rate constant in the dissociation of the hapten–antihapten complex, viz., 10^{-3} sec^{-1} (Levison et al., 1971). While the dissociation rate constant for the fluorescein–antifluorescein complex is 10^{-3}–10^{-6} times those reported for other hapten–antihapten systems, it does compare with dissociation rate constants found for antigen–antibody reactions (Levison et al., 1970). Furthermore, the initial rate of the fluorescein–antifluorescein combination has been shown to obey second-order kinetics over wide ranges in reactant concentrations and the rate constant is as large as that for other hapten–antihapten reactions ($k \sim 10^8$ M^{-1} sec^{-1}) (Levison et al., 1971). Both the high binding affinity of these antifluorescein antibody preparations, together with the sensitivity of the fluroescence measurements on fluorescein, make initial rates easy to measure. It should also be pointed out that antifluorescein antibody, which exhibits both low binding affinity ($K \sim 10^6$–10^8 M^{-1}) and a large degree of heterogeneity with respect to binding, can also be obtained under appropriate immunizing conditions (Dandliker and Feigen, 1961; Dandliker et

† From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037. *Received December 4, 1974.* Supported by Research Grant No. GB-31611 from the National Science Foundation and National Institutes of Health Special Fellowship No. AM42568 S.A.L.

al., 1964; Voss and Lopatin, 1971; Portmann et al., 1975).

This present study focuses on the kinetics of binding between fluorescein antibody which has been obtained at early, intermediate, and late stages of the immune response (Portmann et al., 1975). It further explores how the binding kinetics of antibody (which exhibits homogeneity with respect to equilibrium binding) is influenced by specific anions, both in terms of rate constants and activation parameters. This study is of special interest since it is the first investigation to show specific anion effects on hapten–antihapten reaction kinetics, and is the logical extension of similar salt studies on antigen–antibody kinetics (Levison et al., 1970), as well as on overall antigen–antibody reactions (Dandliker et al., 1967) and overall macromolecular reactions (Von Hippel and Schleich, 1969; Dandliker and de-Saussure, 1971). These studies also indicate that the rate of reaction between fluorescein and antifluorescein, in contrast to early investigations of the Dnp–anti-Dnp reaction, is not diffusion limited and that important structural rearrangements must occur prior to, or during, formation of the hapten–antihapten activated complex.

Kinetic Equations

Initial Rate Method. The initial rate of fluorescence intensity change as previously developed (Levison and Dandliker, 1969) is:

$$dI/dt_0 = k(Q_b - Q_f)(F_{b,max})^{N_1}(F_{f_0})^{N_2} \quad (1)$$

When there is a large quenching of fluorescence upon reaction (Q_f/Q_b is large), appreciable changes in fluorescence polarization occur only when the reaction has already proceeded to a large extent. The usual initial rate conditions no longer hold and a somewhat more complicated polarization equation must be used than that formulated previously (Dandliker and Levison, 1968):

$$\frac{\frac{dp}{dt}\left(\frac{p_b - p_f}{(p_b - p)^2}\right)\frac{Q_f}{Q_b}}{1 + \frac{Q_f}{Q_b}\left(\frac{p - p_f}{p_b - p}\right)} = k(F_{b,max} - F_b)^{N_1}(F_f)^{N_2 - 1} \quad (2)$$

$F_{b,max} - F_b$ and F_f are no longer at their respective zero time concentrations, $F_{b,max}$ and F_{f_0}, but must be calculated from eq 3 or 4 and the value of M. The use of the initial or of the differential rate methods is particularly convenient in that a plot of log initial rate vs. log $(F_{b,max} - F_b)$ at constant F_f, or log initial rate versus log (F_f) at constant $(F_{b,max} - F_b)$, enables the investigator to determine N_1 and N_2 directly in eq 1 or 2 and hence determine the empirical rate laws (Dandliker and Levison, 1968) in an unambiguous manner. In fact, N_1 and N_2 can be obtained from initial rate plots knowing relative concentrations only. N_1 and N_2 have each been determined to be equal to one over extremely wide ranges in reactant concentrations for univalent and divalent antifluorescein systems which have exhibited homogeneity with respect to binding (Levison et al., 1971). We have found that this simple second-order rate behavior is obeyed even for several of the more heterogeneous antifluorescein antibodies studied in this report.

Integrated Rate Method. Various integrated rate expressions have been formulated in terms of fluorescence polarization or intensity parameters, and are listed in Table I. They can be derived by combining classical integrated rate equations (Frost and Pearson, 1961) with the following expressions for the ratio of bound to free fluorescent material (Dandliker et al., 1964):

Table I: Integrated Rate Expressions Involving Fluorescence Polarization and Intensity Parameters.

Expression	Conditions
(1)[a] $\ln\left(\frac{I_\infty - I_0}{I_\infty - I}\right) = k't$, where $k' = k(F_{b,max})_0^{N_1}$	Pseudo-first-order reaction; antibody in large excess; $(F_{b,max})_0$ denotes antibody concentration at zero time
(2)[b] $\ln\left[1 + \left(\frac{Q_f}{Q_b}\frac{(p - p_f)}{(p_b - p)}\right)\right] = k't$, where $k' = k(F_{b,max})_0^{N_1}$	
(3)[c] $\left(\frac{Q_f}{Q_b}\right)\left(\frac{p - p_f}{p_b - p}\right)\left(\frac{1}{(F_{b,max})_0}\right) = kt$	Second-order reaction; equal reactant concentrations; no back reaction
(4) $\left(\frac{I_0 - I}{I - I_\infty}\right)\left(\frac{1}{(F_{b,max})_0}\right) = kt$	
(5) $\left(\frac{1}{F_{b,max} - M}\right)\ln\left\{\left[1 + \frac{Q_f}{Q_b}\left(\frac{p - p_f}{p_b - p}\right)\right]\left[\frac{\left(\frac{M}{F_{b,max}}\right)\left(\frac{Q_f}{Q_b}\right)\left(\frac{p - p_f}{p_b - p}\right)}{1 + \left(\frac{Q_f}{Q_b}\right)\left(\frac{p - p_f}{p_b - p}\right)}\right]\right\} = kt$	Second-order reaction; unequal reactant concentrations; no back reaction; M equals total fluorescein concentration
(6) $\left(\frac{1}{F_{b,max} - M}\right)\ln\left[\frac{(I_\infty - I_0) - (I - I_0)\left(\frac{M}{F_{b,max}}\right)}{(I_\infty - I)}\right] = kt$	

[a] I, I_0, and I_∞, fluorescence intensity at times t, t_0, and t_∞. [b] k, second-order rate constant (M^{-1} sec^{-1}); Q_f/Q_b, ratio of fluorescence intensity of completely free fluorescein to completely bound fluorescein; p, fluorescence polarization at time, t. p of free and completely bound fluorescein are p_f and p_b, respectively. [c] Under these equal reactant concentration conditions M, the total fluorescei fluorescein concentration, equals $F_{b,max}$.

$$\frac{F_b}{F_f} = \frac{Q_f}{Q_b}\left(\frac{p - p_f}{p_b - p}\right) \quad (3)$$

$$\frac{F_b}{F_f} = \frac{Q_f - Q}{Q - Q_b} \quad (4)$$

Results by integrated rate methods have been found to be in agreement with those from the initial rate method.

Materials and Methods

Antibodies. Antifluorescein antisera were obtained from male New Zealand white rabbits weighing approximately 5 lb. Each rabbit was initially inoculated with 4 mg of fluorescein-labeled ovalbumin (dye/protein molar ratio = 0.6) in complete Freund's adjuvant. This amount was administered intradermally at two sites, each site receiving 0.2 ml of the emulsion. Booster injections were given thereafter every 2 months in the same way, using 50 μg of fluorescein–ovalbumin/rabbit. Bleedings were usually taken at weekly intervals at least 10 days after a booster. The antibody studied in this work was obtained at various intervals (up to 17 months) after the initial immunization (Portmann et al., 1975). Sera were stored at 4° after adding 0.1% NaN_3 as a preservative. Immunoglobulin fractions were obtained from sera by precipitating twice with ammonium sulfate at 37.5% saturation, pH 8.3–8.5, and redissolving in 0.15 *M* NaCl. In some cases, immunoglobulins were further purified by chromatography on DEAE-cellulose or by immu-

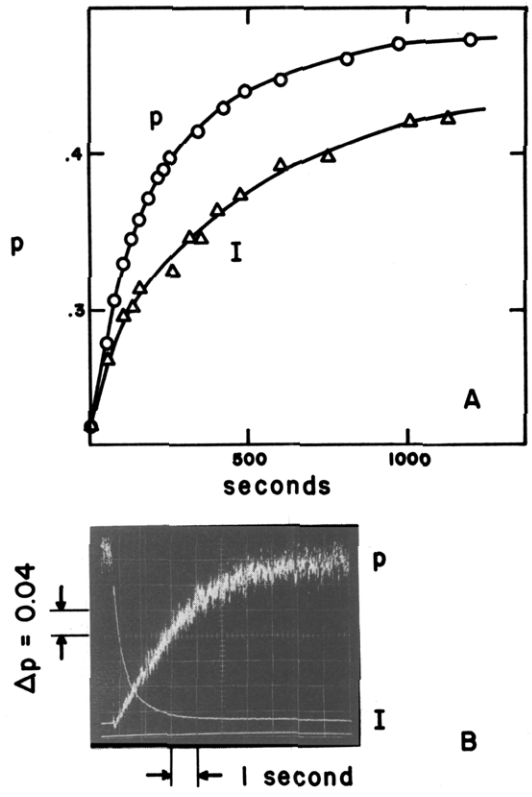

FIGURE 1: Kinetic polarization and intensity curves involving the primary binding of fluorescein to antifluorescein. (A) Fluorescein, 1.9×10^{-9} *M*, and antifluorescein, 4.2×10^{-8} *M* (late, uniformly binding), were hand-mixed within a few seconds in 1 *M* sodium salicylate–0.015 *M* sodium phosphate (pH 7.0) buffer at 14.5° and the fluorescence polarization and enhancement were monitored. The polarization, *p*, is in the units shown, while the intensity, *I*, is in arbitrary units. (B) Stopped-flow trace, fluorescein, 2.5×10^{-8} *M*, and antifluorescein (same as in A), 2.6×10^{-8} *M*, were mixed in 0.15 *M* NaCl–0.015 *M* phosphate at pH 7.0 in the stopped-flow device and the fluorescence polarization, as well as the fluorescence intensity observed as functions of time.

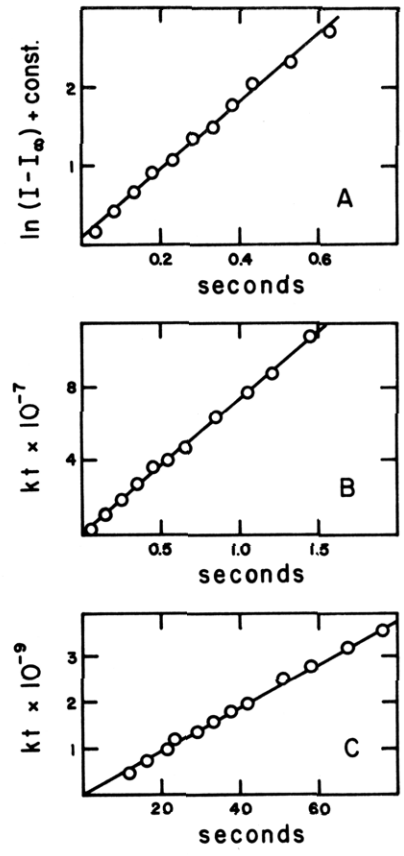

FIGURE 2: Representative integrated rate plots for the fluorescein–antifluorescein reaction in 0.15 *M* NaCl–0.015 *M* phosphate (pH 7.0) at 18.5°. *k* = second order rate constant (M^{-1} sec^{-1}). (A) Fluorescence quenching of fluorescein under pseudo-first-order conditions; antifluorescein (late, uniformly binding), 5.0×10^{-8} *M*, fluorescein, 2.9×10^{-9} *M*; *I* and I_∞ denote fluorescence intensity at times *t* and infinity. The pseudo-first-order rate constant *k'* (sec^{-1}) is obtained from the slope of the plot in A and equals *k* times the initial antibody concentration. (B) Fluorescence quenching, second-order kinetics; antifluorescein, 2.6×10^{-8} *M* (same as in A), fluorescein, 1.5×10^{-8} *M*. For these conditions, eq 5 of Table I becomes: $kt = (1/1.1 \times 10^{-8}) \ln [(I_\infty - I_0) - (I - I_0)0.56]/(I_\infty - I)$, where I_∞, I_0 and *I* denote fluorescence intensity at times infinity, zero, and *t*. (C) Fluorescence polarization, second-order kinetics. Antifluorescein, 2.6×10^{-9} *M* (same as in A and B); fluorescein, 1.5×10^{-9} *M*. For these conditions, eq 6 of Table I was used:

$$kt = \frac{1}{1.1\ 10^{-8}} \ln \left\{ \left[1 + \left(\frac{Q_f}{Q_b} \frac{(p - p_f)}{(p_b - p)} \right) \right] \left[1 - \left(\frac{0.56 \left(\frac{p - p_f}{p_b - p} \right)}{1 + 0.56 \left(\frac{p - p_f}{p_b - p} \right)} \right) \right] \right\}$$

where Q_f/Q_b denotes the ratio of the fluorescence intensity of free to that of completely bound material. p_f and p_b refer to the fluorescence polarization of the free and completely bound material; *p* refers to fluorescence polarization at time *t*.

nospecific purification on fluorescein-substituted cellulose (Levison et al., 1971; Portmann et al., 1971). Fab fragments were prepared from ammonium sulfate fractions by the papain digestion method of Porter (1959). Antibody concentrations were determined by titration of antifluorescein binding activity with fluorescein as described previously (Portmann et al., 1971, 1975), as well as by equilibrium titrations involving appropriate Scatchard plots.

Hapten. Fluorescein was purified chromatographically following the procedure of Dandliker and Alonso (1967). Concentrations were determined by absorption measurements at 490 nm using the extinction coefficient determined previously (Dandliker and Alonso, 1967).

Kinetic Measurements. Measurements in the millisecond to second range were made with a stopped-flow polarimeter whose mixing and stopping times were less than 5 msec (Levison et al., 1971), whereas slower kinetic measurements (10 sec to minutes) were made in a direct readout polarimeter whose time response was of the order of a few seconds (Dandliker and Levision, 1968). Reliable experiments in the millisecond time range could be obtained with fluorescein concentrations as low as 10^{-9} *M* (Levison et al., 1971). The stopped-flow device can measure both fluorescence polarization and intensity simultaneously and is thermostated to ±0.1°. Fluorescein concentrations as low as 5×10^{-11} *M* could be readily measured with the slower device.

In both the manual and stopped-flow measurements, the instrument is first blanked. Then, in the manual device, the fluorescent species is added and mixed in with a footed Teflon rod. Polarization or intensity is recorded as a function of time.

Results

The results presented in this paper fall into several dis-

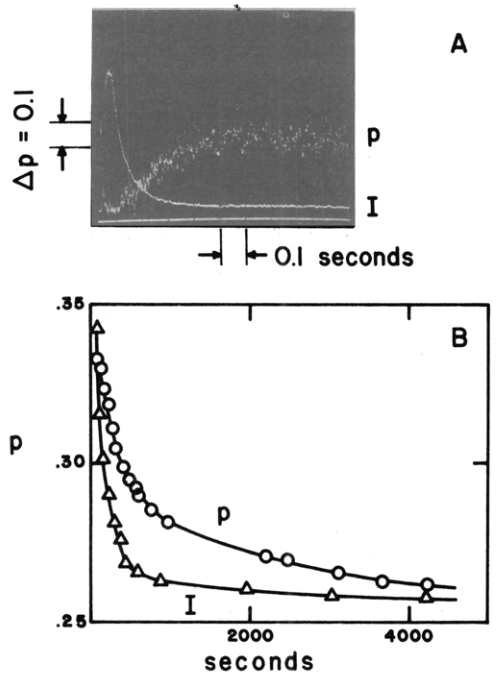

FIGURE 3: Kinetic binding curves, showing two steps in the process of fluorescein binding to antifluorescein. Antifluorescein, 2.0×10^{-7} M (late, $K \sim 10^{11}$ M^{-1}), was mixed with 1.0×10^{-7} M fluorescein, and the rate was studied in 0.15 M NaCl–0.015 M phosphate (pH 7.0) at 18.5°. (A) The primary stage of combination was measured in the stopped-flow device and was completed within 0.3 sec. Increases in fluorescence polarization and quenching of fluorescence were observed. (B) A slower, secondary, presumably conformational change then followed. This slower step had a duration of 1 hr, during which time both the fluorescence polarization and intensity decreased, and was measured in the static fluorescence polarometer. The antibody for this experiment came from a different rabbit than that in Figures 1 and 2.

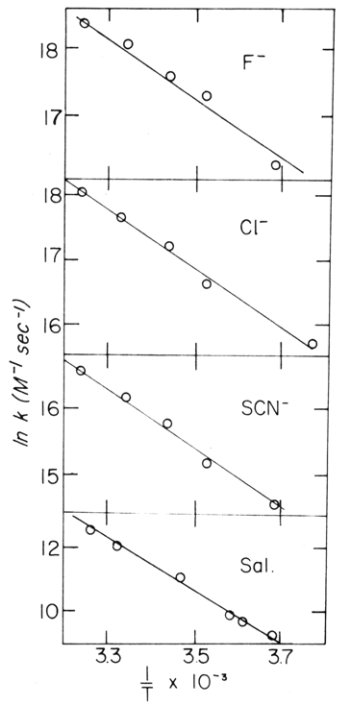

FIGURE 4: Activation energy plots of the second-order rate constant of the fluorescein–antifluorescein reaction in different ionic media. The antibody was the same as that of Figures 1 and 2. All solutions were buffered with 0.015 M phosphate at pH 7.0. Each anion was at a concentration of 1.0 M. Activation energy values (kcal/mol) determined in different media were as follows: salicylate, 15.4; thiocyanate, 10.2; chloride, 8.8; and fluoride, 7.7. In a control experiment, the activation energy was determined to be 7.1 kcal/mol in 0.15 M NaCl.

tinct categories: (1) kinetic binding data by fluorescence polarization or intensity for antibody obtained after long periods of immunization (Figures 1 and 2); (2) forward and second-order rate constants by either initial or integrated rates for antibody obtained after long periods of immunization (univalent and divalent antibody are compared (Table II)); (3) forward and backward rate constants by either initial or integrated rates for antibody obtained after varying periods of immunization (Table IV); (4) evidence for a biphasic reaction for some antibody preparations (Figure 3, Table III); (5) rate and activation parameters for late, divalent antibody in different chaotropic media (univalent and divalent antibody are compared (Figures 4 and 5, Table VI)); (6) rate and activation parameters comparing univalent and divalent antibody (Table V).

Discussion

Typical kinetic curves for fluorescence polarization and intensity changes as functions of time during the course of a binding reaction between fluorescein and its antibody partner, antifluorescein, are shown in Figure 1. An example of a slow reaction is shown in Figure 1A, where the reaction was carried out in the presence of salicylate ion. Under these circumstances, both the fluorescence intensity and polarization increased during the reaction. However, for reactions in the other media studied, the fluorescence intensity is quenched, and the polarization increases during the hapten–antihapten union (Figure 1B). Usually, only one stage was observed in the reaction between fluorescein and antifluorescein. That is, the polarization or intensity underwent a

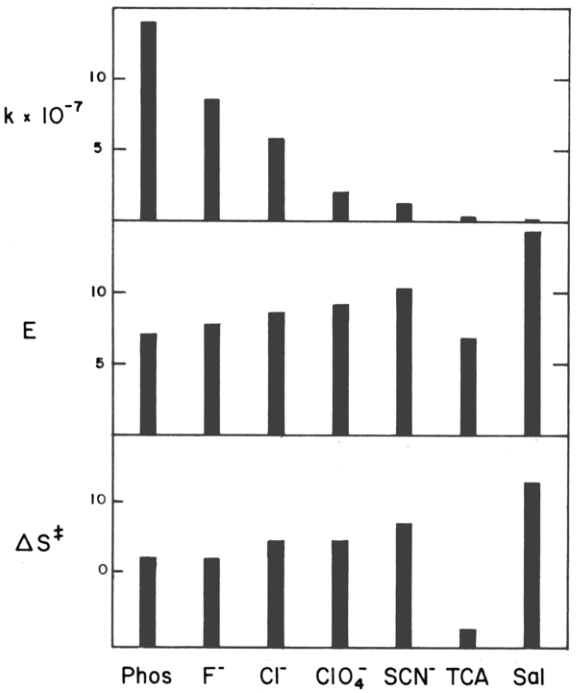

FIGURE 5: Salt effects on the second-order rate constant, k_1 (M^{-1} sec^{-1}), at 18.5°, the activation energy, E_a (kcal/mol) and the activation entropy $\Delta S^{\ddagger}$ (cal/(mol deg)) for the fluorescein–antifluorescein reaction. The antibody here is the same as that of Figures 1, 2, and 4. Each solution contained the appropriate anion at 1.0 M concentration and was buffered with 0.015 M phosphate (pH 7.0). Phos, TCA, and Sal denote phosphate, trichloroacetate, and salicylate ions, respectively. E_a was determined from ln k_1 vs. $1/T$ plots such as shown in Figure 4, whereas $\Delta S^{\ddagger}$ was estimated from k_1 at 18.5° and E_a using the Eyring equation, $\ln k_1 = \ln (k^*T/h) - (\Delta S^{\ddagger}/R) - (E_a/RT)$ (Glasstone et al., 1941).

Table II: Rate Constants for Various Preparations of Late Antifluorescein.

Mode	Type of AB	k_1[d] (M^{-1} sec^{-1})	Concn Range Studied (M)	
			Antibody	Hapten
FP[a] initial rate	Fab[b]	$8.4 \pm 2 \times 10^7$	$2.0 \times 10^{-7} \rightarrow 5.0 \times 10^{-11}$	$5.0 \times 10^{-8} \rightarrow 5.0 \times 10^{-11}$
FQ initial rate	Fab[b]	$8.8 \pm 0.2 \times 10^7$	$2.0 \times 10^{-7} \rightarrow 1.0 \times 10^{-9}$	$5.0 \times 10^{-8} \rightarrow 1.0 \times 10^{-8}$
FQ initial rate	Divalent[b] (immunospecific)	$1.4 \pm 0.2 \times 10^8$	$5.0 \times 10^{-7} \rightarrow 2.0 \times 10^{-9}$	$5.0 \times 10^{-7} \rightarrow 2.0 \times 10^{-8}$
FP initial rate	DEAE[b]	$1.2 \pm 0.2 \times 10^8$	2.0×10^{-8}	1.0×10^{-8}
FQ initial rate, integrated rate	Fab (5723)[c]	$8.3 \pm 0.6 \times 10^7$	$2.0 \times 10^{-8} \rightarrow 1.0 \times 10^{-9}$	$2.0 \times 10^{-8} \rightarrow 1.0 \times 10^{-9}$
FQ initial rate	Divalent (DEAE)[c] (5723)	$1.4 \pm 0.1 \times 10^8$	$5.0 \times 10^{-8} \rightarrow 2.6 \times 10^{-9}$	$1.5 \times 10^{-8} \rightarrow 5.8 \times 10^{-10}$
FQ integrated rate	Divalent (DEAE)[c] (5723)	$1.4 \pm 0.1 \times 10^8$	$5.0 \times 10^{-8} \rightarrow 2.6 \times 10^{-9}$	$1.5 \times 10^{-8} \rightarrow 5.8 \times 10^{-10}$
FQ integrated rate	Divalent (5723)[c] $(NH_4)_2SO_4$ ppt	$1.2 \pm 0.1 \times 10^8$	$2.5 \times 10^{-8} \rightarrow 7.0 \times 10^{-10}$	$2.5 \times 10^{-9} \rightarrow 1.0 \times 10^{-11}$
FQ initial rate	Divalent (serum)[c] (5723)	1.1×10^8	2.5×10^{-8}	4.5×10^{-8}

[a] FP and FQ denote fluorescence polarization and fluorescence quenching, respectively. [b] Pool of four rabbit sera. [c] Individual bleeding of one rabbit (5723). [d] All values of k_1 were determined in 0.15 M NaCl–0.015 M PO_4 (pH 7.0) at 18.5°. In the calculation of k_1, the divalent antibody concentration was assumed to be equal to one-half the binding site concentration, i.e., $F_{b,\,max}/2$. Here k_1 equals twice the value of k determined from equations in Table I.

Table III: Kinetic Parameters Involved in a Two-Step Fluorescein–Antifluorescein Reaction.

Antifluorescein (M)	Fluorescein (M)	k_1[a] (M^{-1} sec^{-1})	k_2 (sec^{-1})
2.3×10^{-8}	1.1×10^{-8}	1.1×10^8	5.0×10^{-3}
2.0×10^{-7}	1.0×10^{-7}	1.0×10^8	5.0×10^{-3}

[a] k_1 is the second-order rate constant for the primary stage of the reaction, while k_2 is the first-order rate constant characteristic of the secondary reaction. All determinations were at 18.5° in 0.15 M NaCl–0.015 M phosphate (pH 7.0).

monotonic change which reached some asymptotic limit at the end of the reaction. Detailed kinetic investigations of this initial process, using the initial and integrated rate methods in the fluorescence polarization or intensity modes, have indicated that a simple second-order rate law is obeyed for the reaction. That is, the reaction is first order with respect to the concentration of each reactant and the value of k is constant over extraordinarily wide ranges of concentration (Table II).

Examples of integrated rate plots in which the equations of Table I were utilized are shown in Figure 2. The plots were linear during at least 90% of the reaction time course, and the determined k values were constant over large changes in the initial concentrations of reactants. The value of the rate constant for divalent antibody is $1.4 \pm 0.1 \times 10^8$ M^{-1} sec^{-1}, while that for univalent Fab preparations is $8.5 \pm 2 \times 10^7$ M^{-1} sec^{-1}. The measured values of these constants are essentially the same regardless of whether the antibody preparation is serum, $(NH_4)_2SO_4$ precipitated, DEAE isolated, or immunospecifically purified, or from which rabbit the antibody was obtained. The magnitude of the rate constants reflects the manner of expressing antibody concentration; these are expressed as molarities regardless of whether univalent or divalent is in question. In the case of divalent antibody, the site concentration is twice that for univalent antibody of the same molarity. Hence, if the sites bound in a purely independent manner, the value of k for divalent antibody would be expected to be twice that of univalent antibody, as was found to be approximately the case.

Finally, it is of interest that the kinetic results obtained by fluorescence polarization agree with those from fluorescence intensity measurements using both initial and integrated rate methods (Table II). The same agreement was previously found for equilibrium studies on the fluorescein–antifluorescein system (Portmann et al., 1975), as well as for equilibrium and kinetic measurements on the dihydrofolate reductase NADPH system (Levison 1975; S. A. Levison, L. E. Gunderson, R. B. Dunlap, F. Otting, A. N. Hicks, and F. M. Huennekens, in preparation).

While most of the antibody preparations studied in this laboratory have exhibited a simple monotonic binding process (Figure 1), preparations from certain individual rabbits showed a biphasic process (Figure 3). The primary stage of reaction under the conditions studied was complete in about 300 msec and involved both fluorescence polarization and quenching changes. A newly observed, slower secondary process followed this fast initial process and was evidenced by a lowering of the polarization and further quenching of the fluorescence intensity. This secondary (presumably conformational) step has a lifetime of the order of 60 min and was characterized by a first-order rate constant which did not change when each of the reactant concentrations were decreased by a factor of 10 (Table III). This biphasic process was also found to occur in the reaction between Fab fragments of this antibody preparation and fluorescein. A similar secondary process has also been found between dihydrofolate reductase and NADPH (Levison, 1975; S. A. Levison, L. E. Gunderson, R. B. Dunlap, F. Otting, A. N. Hicks, F. M. Huennekens, in preparation).

Although the basic theory underlying our quantitative treatment assumes the presence of only two chemical states for the fluorescent-labeled molecule, it seems, nevertheless, justifiable to use the same treatment in following the second phase of the biphasic reaction so long as the first phase is essentially completed before the second phase has proceeded to an appreciable extent.

Investigations of the maturation of the immune response in terms of the binding equilibria between fluorescein and

Table IV: Kinetic and Equilibrium Constants Involved in the Primary Binding of Fluorescein to Antifluorescein Antibody Which was Produced at Early, Intermediate, and Late Stages of the Immune Response.

Time after Initial Injection	Equilibrium Association Constant[a] K (M^{-1}) × 10^{-8}	Heterogeneity Index a	Second-Order Rate Constant k (M^{-1} sec^{-1}) × 10^{-7}	Dissociation Rate Constant[b] k_{-1} (sec^{-1}) × 10^{3}
		Rabbit 5723		
1 month	2.0 ± 0.7	0.65	1.4 ± 0.1	70
6 weeks			2.4 ± 0.5	
2 months	49 ± 20	0.8	3.4 ± 0.2	6.9
5 months	1900	0.9	4.8 ± 0.6	0.25
8 months			5.4 ± 0.5	
12 months	7000 ± 1700	1.0	6.0 ± 0.6	0.09
16 months			6.7 ± 0.4	
17 months	4300 ± 100	1.0	6.6 ± 1.2	0.15
		Rabbit 5724		
2 months	160 ± 30	0.65	5.6	3.5
5 months	~90	0.9	5.5	6.1
12 months	123	0.6	6.7	5.4
17 months	1300 ± 300	0.9	6.7	0.52
		Pool 4 Rabbits		
12 months	630 ± 170	1.0	6.7 ± 0.4	1.1

[a] K was determined by Scatchard equation, $F_b/F_f = K(F_{b,max} - F_b)$. If Scatchard plots were nonlinear, Sips plots were utilized (Portmann et al., 1975). [b] k_{-1} was calculated from the value of k/K. All determinations were at 18.5° in 0.15 M NaCl–0.015 M phosphate (pH 7.0).

antifluorescein antibody produced during early, intermediate, and late stages of the immune response have recently been carried out in our laboratory (Portmann et al., 1971, 1975). It was of further interest to obtain corresponding kinetic data on these systems, by both initial and integrated rate methods (Table IV). Several important facts emerge from a study of this table. First, antifluorescein antibody which exhibits high binding affinity ($K \sim 10^{11}$ M^{-1}) and a restricted degree of heterogeneity has now been isolated from several rabbits and hence confirms earlier discoveries of this nature (Portmann et al., 1971; Levison et al., 1971). This high affinity antibody is produced during late stages of the immune response—about 6 months to 1 year after the initial inoculation. Second, it is the back dissociation rate constant, k_{-1}, rather than the forward association rate constant, which changes dramatically during maturation of the immune response. In one study, k_{-1} decreased by almost a factor of 1000 in magnitude and approached values as low as 10^{-4} sec^{-1} in late stages of the immune response. Since second-order rate behavior was found even for heterogeneous antibody, by both initial and integrated rate measurements, it appears that most of the heterogeneity with respect to binding results from the heterogeneity in the hapten–antihapten dissociation rate. It should be pointed out that there was some curvature in some integrated rate plots involving earlier antibody. This curvature is due to a heterogeneity of the "on" reaction rate constant; the fall-off of k_1 is slight (only a factor of 2–3) and hence is comparable to the change in k_1 found during maturation of the immune response (Table IV). It is obvious that these small changes in k_1 could not account for the observed changes in binding affinity during the progress of the immune response; hence, the latter must be attributed to changes in the rate of the back reaction. Moreover, the antibody concentrations were themselves evaluated by fluorescence titrations (Portmann et al., 1971, 1975) over wide concentration ranges. Antibody titers for sera used in the kinetic work were comparable in magnitude for both early and late antibody. Hence, it is unlikely that the rate measurements could be in error by reason of observing only the behavior of a small segment of the antibody population. Similar indications of the importance of the back reaction have been reported by others (Barisas et al., 1975). This is the first time, however, that it has actually been shown that the "off" reaction rate constant changes dramatically during the course of the immune response.

Table V: Comparison of Kinetic Parameters for Univalent and Divalent Antifluorescein Reacting with Fluorescein.[a]

	k_1 (M^{-1} sec^{-1})[b]	E_a (kcal/mol)[c]	$\Delta S^{\ddagger}$ (cal/(mol deg))[c]
Divalent antibody	1.3 ± 0.1 × 10^8	7.1	2.0
Univalent antibody	8.5 ± 2 × 10^7	7.7	1.8

[a] All experiments carried out in 0.15 M NaCl–0.015 M phosphate (pH 7.0). [b] k_1 refers to second-order rate constant at 18.5°, while E_a refers to the activation energy. $\Delta S^{\ddagger}$, the entropy of activation, was estimated from k_1 and E_a using the Eyring equation (Glasstone et al., 1941). The standard deviations for E_a and $\Delta S^{\ddagger}$ were about ±0.6 kcal/mol and about ±3 cal/(mol deg), respectively. [c] Values calculated for the Dnp–anti-Dnp system from the data of others are: E_a = 4 kcal/mol and $\Delta S^{\ddagger}$ = −11 cal/(mol deg) (Levison et al., 1968).

The antifluorescein antibody obtained from one rabbit (5723) after 1 year following the initial inoculation was of special interest because of its extremely high association constant (7 × 10^{11} M^{-1}), its apparent homogeneity with respect to binding, and its large second-order rate constant, whose value (1.4 × 10^8 M^{-1} sec^{-1}) approaches the limiting value of other second-order rate constants reported for hapten–antihapten systems (Day et al., 1963). In many ways, this fluorescein–antifluorescein system represents a model, simple hapten–antihapten reaction, devoid of the inherent ambiguities associated with low-affinity, heterogeneous antibody. Detailed studies of the effect of temperature on the second-order rate constant for this high affinity, uniformly binding antibody have revealed some important observations. As indicated in Table V, the activation energy for the forward reaction is in the 7–8-kcal/mol range, regardless of whether divalent antibody or univalent Fab fragments are used. These values are, as in the case of antigen–antibody reactions (Levison et al., 1968), somewhat higher than the 4-kcal/mol value expected for a diffusion controlled reaction (Longsworth, 1954) and the 4-kcal/mol value reported for Dnp–anti-Dnp systems (Day et al., 1963; Barisas et al., 1975). Furthermore, as in the case of antigen–antibody reactions (Levison et al., 1968), the entropy of activation for the fluorescein–antifluorescein reaction has been found also to be more positive than that for the Dnp–anti-Dnp system and for many other reactions in solution in which two molecules combine to form one molecular entity (Laidler, 1965). It may be noted that the effects of the entropy and enthalpy of activation on the rate of the fluorescein–antifluorescein reaction are in opposite directions, tend somewhat to compensate, but still allow for a very fast reaction.

In studies of anion effects on antigen–antibody kinetics (Levison et al., 1970), it was found that the value of anti-

Table VI: Salt Effects on Rate and Activation Parameters of the Fluorescein–Antifluorescein Reaction.

Anion[a]	k_1[b] (M^{-1} sec^{-1}) (18.5°) × 10^{-7}	E_a (kcal/mol)	$\Delta S^{\ddagger}$[c] (cal/(mol deg))
Phosphate	14	7.4	2.2
Fluoride	8.6	7.7	2.0
Chloride	5.8	8.7	4.7
Perchlorate	1.9	9.3	4.5
Thiocyanate	1.3	10.2	7.0
Trichloroacetate	0.26	6.8	−8.0
Salicylate	0.0130	14.6	12.8

[a] All experiments were carried out in 1 M sodium salt concentrations in 0.015 M phosphate (pH 7.0). [b] k_1 refers to second-order rate constant at 18.5°, where E_a refers to experimental activation energy. [c] $\Delta S^{\ddagger}$ refers to the entropy of activation and was estimated from k_1 at 18.5° and E_a using the Eyring equation (Glasstone et al., 1941). (See Definition of Symbols.) Standard deviations range from 0.5 to 1.0 kcal/mol for E_a and from 2 to 4 cal/(mol deg) for $\Delta S^{\ddagger}$.

gen–antibody second-order rate constants in various media increases according to the Hofmeister sequence of ions: $SCN^- < ClO_4^- < Cl^- < F^- < SO_4^{2-} <$ phosphate. Hence, the rate of primary binding of antigen to antibody in chaotropic media (SCN^-, ClO_4^-) is markedly lower than in nonchaotropic media (phosphate, sulfate). We have now extended this approach to hapten–antihapten reactions and have studied the effect of anions on the second-order rate constant, and also upon the activation energies and entropies. The significance of measuring activation parameters of a hapten–antihapten reaction or, for that matter, any second-order macromolecular reaction in different ionic media has, to our knowledge, not previously been appreciated. A survey of second-order rate constants and activation parameters derived from Arrhenius plots (Figure 4) is given in Figure 5 and Table VI. Again, there are profound anion effects which follow the trends observed initially for antigen–antibody second-order rate constants (Levison et al., 1970). The changes correlate with the Hofmeister series, the second-order rate constant decreases as follows: phosphate > F^- > Cl^- > ClO_4^- > SCN^- > trichloroacetate > salicylate. The changes are large; k for phosphate is about 10^3 times as great as that of salicylate, while E_a for phosphate is over 7 kcal/mol smaller than that for salicylate. The activation energy decreases as follows: salicylate > SCN^- > ClO_4^- > Cl^- > F^- ≃ phosphate. Estimates of the activation entropy indicate that this parameter decreases as follows: salicylate > SCN^- > ClO_4^- > Cl^- > F^- > phosphate > trichloroacetate. One anion, trichloroacetate, seems atypical, in that a lowered k is accompanied by a relatively low activation energy and a low entropy of activation. Although the standard deviation in determining activation entropies is large (±4 cal mol^{-1} deg^{-1}), the trends shown in Figure 5 and Table VI are significant. Furthermore, we have found that high concentrations of nonchaotropic anions (1 M phosphate) do not produce fractional orders with respect to divalent antifluorescein even at low temperatures. This is in contrast to results reported for the binding kinetics of antigen–antibody reactions (Levison and Dandliker, 1969; Levison et al., 1970), where a fractional order with respect to divalent antibody was found in nonchaotropic media. We propose a mechanism by which fluorescein and antifluorescein undergo a simple bimolecular process to form a primary hapten–antihapten complex:

$$\text{AB} + \text{hapten} \xrightarrow{k_1} \text{AB}\cdots\text{hapten} \quad (5)$$

where antibody and/or hapten undergo some sort of structural rearrangement prior to or during combination. To account for the slow, first-order process (time constant ~ several minutes) found with some antibody preparations, it is reasonable to postulate a slow, unimolecular process following the initial primary complex formation:

$$\text{AB}\cdots\text{hapten} \xrightarrow{k_2} \text{AB–hapten} \quad (6)$$

The nature of this secondary process is not known, but probably involves further changes in hydrophobic bonding, solvent loss, or protein conformation. Interpretation of electron micrographs (Feinstein and Rowe, 1965; Valentine and Green, 1967) supports the idea that the antibody molecule clicks open during combination with a hapten or antigen partner and acquires an open rod-like shape as compared to a more globular form prior to reaction. There are two equivalent, alternative ways in which the supposed structural rearrangements may be discussed. One is that this rearrangement involves solvent reorganization and loss and/or unfolding during formation of the transition state. The idea of solvent loss is suggested by the fact that estimates of the entropy of activation indicate much more positive values than those determined for other bimolecular reactions (Laidler, 1965). Furthermore, one can argue that if the activated complex is less solvated than the isolated reactants, then anions which compete more effectively for solvent water molecules will tend to promote the rate of reaction. This is what is observed experimentally. Anions with high charge densities (e.g., phosphate) tend to enhance the reaction rate, whereas anions with low charge densities (e.g., thiocyanate) tend to lower the reaction rate. Solvation effects are important in many organic SN2 reactions in protic and aprotic solvents (Parker, 1967), as well as in the formation of final antigen–antibody complexes (Haurowitz, 1952). Similar salt effects on equilibrium association constants of *p*-azobenzoate hapten–anti-*p*-azobenzoate hapten (Pressman et al., 1961) and of several antigen–antibody reactions (Dandliker et al., 1967) have previously been reported. Furthermore, most antigen–antibody systems which have been studied by one method or another during the last several years appear to have overall entropies of reaction which are more positive than would be expected on the basis of the loss of translational and rotational motion by the reactants as they form a final antigen–antibody complex (Boyd, 1956). A discussion of specific anion effects on reaction rates can also be based upon the concept of hydrophobic bonding. Highly chaotropic ions such as ClO_4^- and SCN^- render hydrophobic interactions weaker. If such interactions are involved in the formation of the transition state complex, then the rate of the forward reaction may be expected to be lowered in highly chaotropic media. Effects of chaotropic ions on the structure of the reactants themselves with a consequent change in the free energy of activation would give similar results and cannot presently be ruled out. The above considerations tie in with the well-known "dissociating" effect of chaotropic media on antigen–antibody complexes (Dandliker et al., 1967). The presence of chaotropic ions impedes the process of combination, but has relatively little effect on the rate of dissociation of antigen–antibody complexes (Levison et al., 1970). Probably the same is true of hapten–antihapten complexes in view of the common difficulty encountered in attempting to dissociate high avidity hapten–antihapten complexes. The molecular

picture suggested by these phenomena is that chaotropic ions can act primarily only when reactants are separated, but once they are firmly bound, and solvent and ions have been squeezed out, there is no obvious means by which the chaotropic ions can exert a direct effect. Obviously, an effect on the forward rate alone is sufficient to produce an observed lowering of the equilibrium constant. The effects of chaotropic ions on hydrophobic bonding are of course superimposed on the weakening of hydrogen bonds and electrostatic interactions which would be produced nonspecifically by high concentrations of any ion. Also, effects may occur due to specific ion interactions with the reactants. A case in point is provided by the behavior of the fluorescein–antifluorescein reaction in phosphate vs. salicylate. The fluorescence of fluorescein is quenched markedly by salicylate as compared to phosphate or most other ions. In 1 M phosphate, the formation of the fluorescein–antifluorescein complex is accompanied by a large quenching (Q_f/Q_b = 16). However, in 1 M salicylate, there is actually an enhancement (Q_f/Q_b = 0.25). This somewhat anomalous behavior may be evidence of a close interaction between fluorescein and salicylate. The fluorescence enhancement occurring when fluorescein reacts with antibody is then consistent with breakup of the fluorescein–salicylate interaction. Here again, it appears that the effect of ions is primarily on the isolated reactants since the fluorescence intensity of the fluorescein–antifluorescein complex is nearly the same in the presence or absence of salicylate.

A reaction mechanism consisting of eq 5 and 6 closely resembles that previously proposed for antigen–antibody reactions (Levison and Dandliker, 1969; Levison et al., 1970), and more recently proposed for MOPC-315 myeloma–Dnp interactions (Haselkorn et al., 1974). For antigen–antibody reactions, our scheme postulated the rapid, reversible formation of a loosely held antigen–antibody encounter pair, the formation of which was not necessarily diffusion controlled (as was supposed by Haselkorn et al., 1974, in their mechanism). If one prefers by matter of taste to define the primary reaction as "collision", then few would argue that this reaction is necessarily diffusion controlled. However, the physical phenomena utilized in this work are not measuring collision rates, but instead some chemical phenomena subsequent to "collision". To account for the fractional order of reaction with respect to divalent antibody in nonchaotropic media, it was necessary to postulate also that there was a distribution (such as Sipsian) of binding free energies in the encounter pair formation and that the steady-state encounter pair concentration was very low. In the case of the fluorescein–antifluorescein (hapten–antihapten) reaction, no fractional orders of reaction have ever been observed, but nevertheless, two distinct steps in complex formation have been found for some preparations. Because of the uniformity in the binding sites of the antibody studied in the present work, fractional orders of reaction would not be expected within the framework of the encounter pair mechanism previously proposed (Levison and Dandliker, 1969).

The existence of antifluorscein antibodies with association constants of the order of 10^{11} poses some interesting theoretical questions concerning the maxium possible value of an association constant for a hapten–antihapten interaction. It is evident that the strength of this interaction has an upper limit which is dependent upon interactions between the various amino acid residues in the H and L chains (which are limited in size between comparatively narrow ranges) and the "complementary" groups of the hapten. As was pointed out some time ago (Karush, 1962; Dandliker et al., 1967), the predominant kinds of interactions which contribute to the stability of antigen–antibody or hapten–antibody complexes are electrostatic forces, hydrogen bonds, hydrophobic bonding, and weak dispersion forces. The interaction free energy between an antibody site and the antigen or hapten is a net effect determined by the number and type of these interactions which can be brought into play as the interacting molecules collide, stick, and then slowly rearrange to give the complex of minimum free energy. As an example of how these principles apply, we have found that high affinity antifluorescein ($K \sim 10^{12}$) shows no measurable tendency to combine with rhodamine B, even at 5×10^{-5} M antibody. This fact has some important implications for the hapten–antibody interaction. In the most fundamental sense, all such interactions are completely determined by the three-dimensional distribution of electron density in the antibody site and in the hapten determinant. The structure of fluorescein differs from that of rhodamine mostly in the presence of the negatively charged phenoxide ion and the quinoid-like oxygen which give peaks in the local and hence specific electron density distribution, whereas in rhodamine, at the geometrically corresponding locations the peaks have disappeared and have perhaps become valleys (as at the positively charged diethylammonium group). Because of the greater importance of monopolar forces as compared to dipolar or multipolar forces, it is the differences in size and charge distribution to which the differences in binding affinities and specificities should be attributed.

Definition of Symbols

e, equilibrium value of parameter
f,b, free and bound forms, respectively, of fluorescein
0, at time approaching 0,
AB, divalent antibody molecule
E_a, experimental activation energy, kcal/mol
Fab, univalent antibody molecule
$F_{b,max}$, the maximum number of sites available for antibody
F_b, molar concentration of fluorescein in bound form
F_f, molar concentration of fluorescein in free form
I, fluorescence intensity
k, empirical second-order rate constant (M^{-1} sec^{-1}), defined by eq 1 and 2
k_1, second-order rate constant (M^{-1} sec^{-1}), whose value is twice that of k when it is expressed in rate equations utilizing the molarity of divalent antibody. Defined by eq 5:

$$\text{AB} + \text{hapten} \xrightarrow{k_1} \text{AB}\cdots\text{hapten}$$

k_{-1}, back dissociation rate constant (sec^{-1}) defined by

$$\text{AB–hapten} \xrightarrow{k_{-1}} \text{hapten} + \text{AB}$$

K, equilibrium association constant for hapten–antibody site binding as defined by Scatchard equation:

$$F_b/F_f = K(F_{b,max} - F_b)$$

k', (sec^{-1}) pseudo-first-order rate constant defined by eq 1 and 2 in Table I
k_2, (sec^{-1}) first-order rate constant involved in final AB–hapten complex formation
M, the sum of $F_b + F_f$
N_1, order of reaction with respect to AB or F_{ab} defined by eq 1 and 2

N_2, order of reaction with respect to fluorescein defined by eq 1 and 2

p, polarization of fluorescence

Q, ratio of fluorescence intensity to molar concentration of fluorescein

$\Delta S^{\ddagger}$, activation entropy, related to E_a by Eyring equation (Glasstone et al., 1941):

$$\ln k_1 = \frac{k^* T}{h} + \frac{\Delta S^{\ddagger}}{R} - \frac{E_a}{RT}$$

where h = Planck's constant, k^* = Boltzmann's constant, T = absolute temperature, and R = gas constant

(dI/dt), rate of change of fluorescence intensity

(dp/dt), rate of change of polarization

References

Barisas, B. G., Singer, S. J., and Sturtevant, J. M. (1975) *Immunochemistry 12,* 411.

Boyd, W. C. (1956), Fundamentals of Immunology, 3rd ed, New York, N.Y., Interscience, p 285.

Dandliker, W. B., and Alonso, R. (1967), *Immunochemistry 4,* 191.

Dandliker, W. B., Alonso, R., deSaussure, V. A., Kierszenbaum, F., Levison, S. A. and Schapiro, H. C. (1967), *Biochemistry 6,* 1460.

Dandliker, W. B., and deSaussure, V. A. (1971), The Chemistry of Biosurfaces, Vol. 1, Hair, M. L., New York, N.Y., Marcel Dekker, p 1.

Dandliker, W. B., and Feigen, G. A. (1961), *Biochem. Biophys. Res. Commun. 5,* 299.

Dandliker, W. B., and Levison, S. A. (1968), *Immunochemistry 5,* 171.

Dandliker, W. B., Schapiro, H. C., Meduski, J. W., Alonso, R., Feigen, G. A., and Hamrick, J. R., Jr. (1964), *Immunochemistry 1,* 165.

Day, L. A., Sturtevant, J. M., and Singer, S. J. (1963), *Ann. N.Y. Acad. Sci. 103,* 611.

Feinstein, A., and Rowe, A. J. (1965), *Nature (London) 205,* 147.

Froese, A. (1968), *Immunochemistry 5,* 253.

Froese, A., Sehon, A. H., and Eigen, M. (1962), *Can. J. Chem. 40,* 1786.

Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, 2nd ed, New York, N.Y., Wiley, p 186.

Glasstone, S., Laidler, K. J., and Eyring, H. (1941), The Theory of Rate Processes, New York, N.Y., McGraw-Hill, p 169.

Haselkorn, D., Friedman, S., Givol, S., and Pecht, I. (1974), *Biochemistry 13,* 2210.

Haurowitz, F. (1952), *Biol. Rev. Cambridge Philos. Soc. 27,* 247.

Karush, F. (1962), *Adv. Immunol. 2,* 1.

Kelly, K. A., Sehon, A. H., and Froese, A. (1971), *Immunochemistry 8,* 613.

Laidler, K. J. (1965), Chemical Kinetics, New York, N.Y., McGraw-Hill, p 198.

Levison, S. A. (1975), Biochemical Fluorescence; Concepts, Vol. I, Chen, R. F., and Edelhoch, H., Ed., New York, N.Y., Marcel Dekker, p 375.

Levison, S. A., and Dandliker, W. B. (1969), *Immunochemistry 6,* 253.

Levison, S. A., Jancsi, A. N., and Dandliker, W. B. (1968), *Biochem. Biophys. Res. Commun. 33,* 942.

Levison, S. A., Kierszenbaum, F., and Dandliker, W. B. (1970), *Biochemistry 9,* 322.

Levison, S. A., Portmann, A. J., and Dandliker, W. B. (1971), *Biochem. Biophys. Res. Commun. 43,* 258.

Longsworth, L. G. (1954), *J. Phys. Chem. 58,* 770.

Pecht, I., Givol, D., and Sela, M. (1972), *J. Mol. Biol. 68,* 241.

Porter, R. R. (1959), *Biochem. J. 73,* 119.

Portmann, A. J., Levison, S. A., and Dandliker, W. B. (1971), *Biochem. Biophys. Res. Commun. 43,* 207.

Portmann, A. J., Levison, S. A., and Dandliker, W. B. (1975), *Immunochemistry* (in press).

Pressman, D., Nisonoff, A., and Radzimski, G. (1961), *J. Immunol. 86,* 35.

Valentine, R., and Green, N. (1967), *J. Mol. Biol. 27,* 615.

Von Hippel, P. H., and Schleich, T. (1969), Structure and Stability of Biological Macromolecules, New York, N.Y., Marcel Dekker, p 417.

Voss, D. E., and Lopatin, E. W. (1971), *Biochemistry 10,* 208.