Salt Effects on Antigen–Antibody Kinetics*

S. A. Levison, F. Kierszenbaum, and W. B. Dandliker

ABSTRACT: The influence of anions on the reaction kinetics of the primary combination occurring between fluorescent-tagged antigen and antibody molecules has been investigated at 1.5 ± 0.5° in pH 7.0 buffer solutions. Fluorescence polarization techniques have been applied to kinetic studies of three different antigen–antibody systems: fluorescein-labeled ovalbumin–antiovalbumin, dansyl-labeled bovine serum albumin–antibovine serum albumin, and fluorescein-labeled γ-globulin–antifluorescein. In all three cases, it has been shown that the initial rate of reaction is markedly influenced by the nature of the ionic medium and that an interesting relationship between the Hofmeister series and the empirical initial rate law exists. Specifically, for antigen divalent antibody systems, chaotropic ions, which promote macromolecular unfolding and dissociation, correlate with a simple second-order rate law, whereas nonchaotropic ions, which promote macromolecular folding and association, correlate with an initial rate law involving a fractional order with respect to antibody concentration. However, kinetic studies of antigen–Fab (univalent) antibody systems have shown that a simple second-order initial rate law is followed in both chaotropic and nonchaotropic environments. These results indicate that during the initial stages of reaction, whole antibody and Fab antibody react with antigens by different mechanisms in nonchaotropic media. Finally, it is concluded that a general correlation of the magnitude of all second-order rate constants determined in this report with the Hofmeister series exists. In particular, the value of the second-order rate constant in various media increases according to the following sequence of ions: $SCN^- < ClO_4^- < Cl^- < F^- < SO_4^{2-} <$ phosphate. Mechanisms consistent with these observations and which imply important structural rearrangements involving the loss of water prior to, and during the initial combination between antigen and antibody are proposed.

Symbols

Subscripts

e, equilibrium value of parameter
f, b, free and bound forms, respectively, of fluorescent-labeled material
0, at time approaching zero
(AB), stoichiometric molar concentration of antibody
(AG), stoichiometric molar concentration of antigen
k, defined by $-\frac{d(AG)}{dt} = k(AB)^{N_1}(AG)^{N_2}$, eq 3
k_1, bimolecular rate constant defined by eq 6
k_{-1}, unimolecular rate constant defined by eq 6
K, $\frac{k_1}{k_{-1}}$ equals equilibrium association constant defined by eq 6
k', empirical rate constant defined by eq 7
k'', empirical rate constant defined by eq 8
k_p, unimolecular rate constant defined by eq 11
N_1, order of reaction with respect to (AB)
N_2, order of reaction with respect to (AG)
p, polarization of fluorescence
Q, ratio of fluorescence intensity to molar concentration of fluorescent-labeled material
$\left(\frac{dp}{dt}\right)$, rate of change of polarization

It is well known that the Hofmeister series describes in a general manner the order of effectiveness of the influence of specific ions on macromolecular structures (Hamaguchi and Geiduschek, 1962; von Hippel and Wong, 1964; Robinson and Jencks, 1965). While there have been several investigations which have correlated enzyme steady-state activity with this series (Fridovich, 1963; Warren *et al.*, 1966; Seidel, 1969), practically no data are available concerning how these salts influence the binding kinetics of two macromolecules such as antigen and antibody. The development of fluorescence polarization kinetic techniques (Dandliker and Levison, 1967) has afforded us a unique opportunity to study the effects of these salts on the primary combination between macromolecules (Levison *et al.*, 1969). Recently it has been shown that the reaction binding kinetics between fluorescein-labeled ovalbumin and divalent antiovalbumin obey a rate law which is significantly different in buffers containing phosphate, compared with those containing Tris (Levison and Dandliker, 1969). We have now undertaken a detailed study of the effects of several anions in different regions of the Hofmeister series on the kinetics of the primary combination for three antigen–antibody systems: fluorescein-labeled ovalbumin–antiovalbumin, dansyl-labeled bovine serum albumin–anti bovine serum albumin, and fluorescein-labeled γ-globulin–antifluorescein. These kinetic studies include comparisons between γG[1] antibody (divalent) and its Fab (univalent) fragments.

This particular report demonstrates that a definite correlation exists between the Hofmeister series and the kinetics of

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[1] Abbreviations used are: γG, γ-globulin; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

the antigen–antibody systems studied. The results suggest that important molecular rearrangements occur prior to the combination process and that the formation of the transition state for the antigen–antibody complex probably involves both the loss of solvent molecules as well as an unfolding of the macromolecules involved.

Materials and Methods

Rabbit antisera was made as described previously (Kierszenbaum and Dandliker, 1968). The γ-globulin fraction was obtained by ammonium sulfate precipitation (Campbell *et al.*, 1963) and further purified as follows: Rabbit antiovalbumin was purified by DEAE-cellulose chromatography (Campbell *et al.*, 1963), whereas rabbit anti bovine serum albumin was purified by Sephadex G-200 chromatography (Kierszenbaum *et al.*, 1969a). Univalent Fab fragments were prepared from γG antibody by papain digestion (Porter, 1959). Antifluorescein antibody was prepared by immunizing rabbits with fluorescein-labeled ovalbumin as described previously (Dandliker *et al.*, 1964).

Both dansyl-labeled bovine serum albumin and fluorescein-labeled ovalbumin were prepared by preparative acrylamide gel electrophoresis and gel filtration on Sephadex G-100, respectively (Kierszenbaum *et al.*, 1969a,b). Narrow fractions of each were obtained from the center portions of the peaks. The dye to protein molar ratio for dansyl bovine serum albumin was about 2.7, whereas for fluorescein–ovalbumin it was about 0.7. The test antigen for antifluorescein activity was γG labeled with fluorescein which had a dye to protein ratio of 0.45. Typical polarization values of free, uncombined, and labeled antigens in solutions containing 0.15 M NaCl, 0.01 M Na_2HPO_4, and 0.005 M NaH_2PO_4 at 1.5 ± 0.5° are as follows: dansyl bovine serum albumin, 0.292; fluorescein–ovalbumin, 0.327; and fluorescein–γG, 0.305.

Antibody concentrations were measured by precipitin determinations as described previously (Dandliker and Levison, 1967). The procedure involving kinetic measurements utilized a direct readout fluorescence polarimeter (Dandliker and Levison, 1967; J. White *et al.*, 1969, unpublished data). All pH measurements were made with a Radiometer pH meter at 23°.

Antigen and antibody preparations were stored frozen in small portions and thawed immediately prior to use. Only one preparation of antigen and of antibody was used in studying salt effects on the kinetics of a particular antigen–antibody system.

Kinetic Equations

The kinetic studies employed both initial rate and integrated rate methods utilizing fluorescence polarization parameters as previously developed (Dandliker and Levison, 1967). The method of initial rates makes use of the following equations: For constant $(AB)_0$ but varying $(AG)_0$

$$\log\left[\left(\frac{dp}{dt}\right)_0\right] = (N_2 - 1)\log[(AG)_0] + \text{constant}_a \quad (1)$$

$$\text{where} \quad \text{constant}_a = \log\left[\frac{Qb}{Qf}(p_b - p_f)\,k(AB)_0^{N_1}\right]$$

For constant $(AG)_0$ but varying $(AB)_0$

$$\log\left[\left(\frac{dp}{dt}\right)_0\right] = N_1 \log[(AB)_0] + \text{constant}_b \quad (2)$$

$$\text{where} \quad \text{constant}_b = \log\left[\frac{Qb}{Qf}(p_b - p_f)\,k(AG)_0^{N_2-1}\right]$$

(dp/dt_0) refers to the rate of change of polarization as $t \to 0$. N_1 and N_2 are defined by a rate law of the following form:

$$-\left(\frac{d(AG)}{dt}\right)_{t\to 0} = k(AB)_0^{N_1}(AG)_0^{N_2} \quad (3)$$

or, in terms of polarization changes:

$$\left(\frac{dp}{dt}\right)_0 = \frac{Qb}{Qf}(p_b - p_f)k(AB)_0^{N_1}(AG)_0^{N_2-1} \quad (4)$$

where k is the usual empirical rate constant and is defined by eq 3.

The method of integrated rates utilizes a previously derived relationship for pseudo-first-order reactions (Dandliker and Levison, 1967):

$$\log(p_e - p) = \log(p_e - p_f) - \left[\frac{k_1(AB)^{N_1} + k_{-1}}{2.3}\right]t \quad (5)$$

where p refers to the polarization at time t and p_f refers to the polarization of the free, unbound antigen. The polarization of the system at equilibrium is denoted by p_e. The forward association and dissociation rate constants, k_1 and k_{-1}, respectively, are defined in terms of the following reaction:

$$\mathfrak{F} + \mathfrak{R} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathfrak{F}\mathfrak{R} \quad (6)$$

where $\mathfrak{F}$ is a fluorescent-tagged antigen molecule, $\mathfrak{R}$ is a receptor antibody site, and $\mathfrak{F}\mathfrak{R}$ is the antigen–antibody complex. The pseudo-first-order rate constant, equal to $k_1(AB)^{N_1}$, is assumed to be constant during the reaction since $(AB)_0 \gg (AG)_0$.

In excess AB concentration, plots of $\log(p_e - p)$ *vs.* time have been shown to be linear, at least up to the half-life of the polarization change (Dandliker and Levison, 1967) and yield the parameter, $k_1(AB_0^{N_1} + k_{-1})/2.3$, denoted by S. (The deviation from pseudo-first-order behavior after the half-time may be indicative of site nonuniformity, site depletion, or possibly a change in the reaction mechanism.) Plots of S *vs.* $(AB)_0$ are linear [provided the order with respect to (AB) equals one] and yield the important parameters k_1 and k_{-1} (from the slope and the intercept, respectively).

Results

Effect of Specific Ions on the Empirical Rate Law for the Antigen–Divalent Antibody Combination. Initial rate measurements, employing eq 1 and 2, were utilized to ascertain the form of the rate law in various ionic media for three different antigen–antibody systems: fluorescein-labeled ovalbumin–

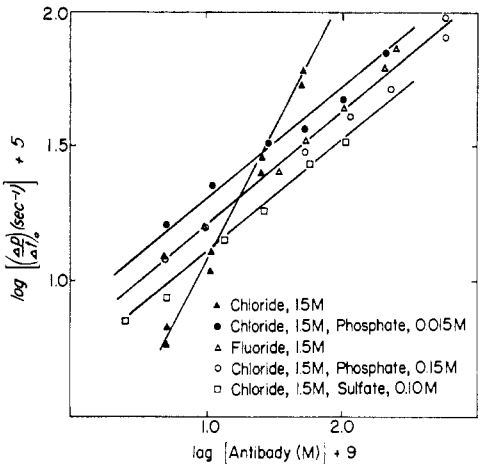

FIGURE 1: Effect of nonchaotropic ions on the order of reaction with respect to antibody concentration for the fluorescein-labeled ovalbumin–divalent antiovalbumin system (see eq 2). All of the studies were performed in pH 7.0 solutions at 1.5 ± 0.5°. The antigen was a narrow fraction of fluorescein-labeled ovalbumin, purified on Sephadex G-100. The antibody was an IgG preparation isolated by DEAE-cellulose chromatography. Similar results were obtained with immunospecifically purified antibody (Levison and Dandliker, 1969): (▲) 1.5 M KCl–0.01 M Tris; (□) 1.5 M KCl–0.1 M K_2SO_4–0.01 M Tris; (△) 1.5 M KF–0.01 M Tris; (●) 1.5 M KCl–0.01 M K_2HPO_4–0.005 M KH_2PO_4; (○) 1.5 M KCl–0.01 M K_2HPO_4–0.05 M KH_2PO_4.

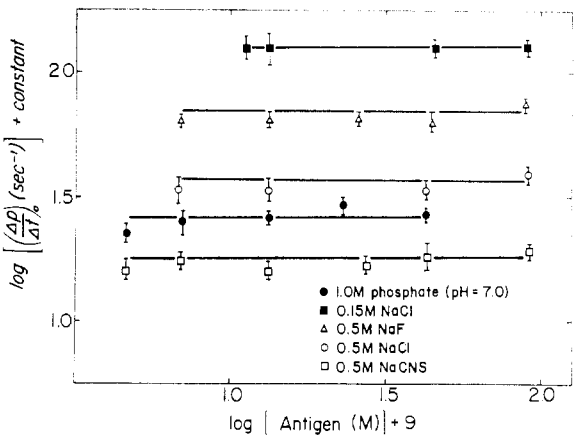

FIGURE 3: Determination of the order of reaction with respect to antigen for the dansyl-labeled bovine serum albumin–divalent anti bovine serum albumin system in various ionic media at 1.5 ± 0.5° (see eq 1). All solutions were buffered at pH 7.0: (●) 1.0 M phosphate; (■) 0.15 M NaCl–0.01 M Tris; (△) 0.5 M NaF–0.01 M Tris; (○) 0.5 M NaCl–0.01 M Tris; (□) 0.5 M NaCNS–0.01 M Tris.

antiovalbumin, dansyl-labeled bovine serum albumin–anti bovine serum albumin and fluorescein-labeled γG–antifluorescein.

Effects of relatively nonchaotropic ions, *e.g.*, phosphate, sulfate, and fluoride, were contrasted with those of more highly chaotropic ions such as chloride, perchlorate, and thiocyanate. Typical plots of log $[(dp/dt)_0]$ *vs.* log $[(AB)_0]$ at constant $(AG)_0$ as shown in Figures 1 and 2, were used to determine N_1, the order of reaction with respect to (AB) for the ovalbumin–divalent antiovalbumin and bovine serum albumin–divalent anti bovine serum albumin systems. An example of the determination of the order with respect to (AG), N_2, is shown in Figure 3 for the bovine serum albumin–divalent antibovine serum albumin system where log $[(dp/dt)_0]$ is plotted against log $(AG)_0$ at constant $(AB)_0$. The values of N_1 and N_2 for both of these determinations as well as other N_1, N_2 values, which include those obtained for the fluorescein-labeled γG–divalent antifluorescein combination are listed in Tables I and II. These results clearly show in all three cases, that when antigen and divalent antibody react in ionic media composed of chao-

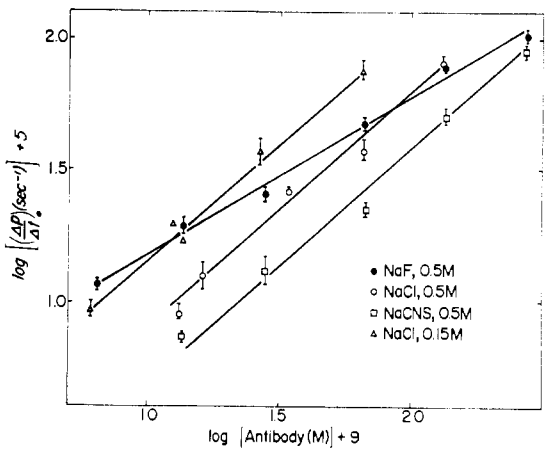

FIGURE 2: Determination of the order of reaction with respect to antibody concentration for the dansyl-labeled bovine serum albumin–divalent anti bovine serum albumin system in various ionic media (see eq 2). All of the studies were performed in pH 7.0, 0.01 M Tris solutions at 1.5 ± 0.5°: (●) 0.5 M NaF; (○) 0.5 M NaCl; (□) 0.5 M NaCNS; (△) 0.15 M NaCl.

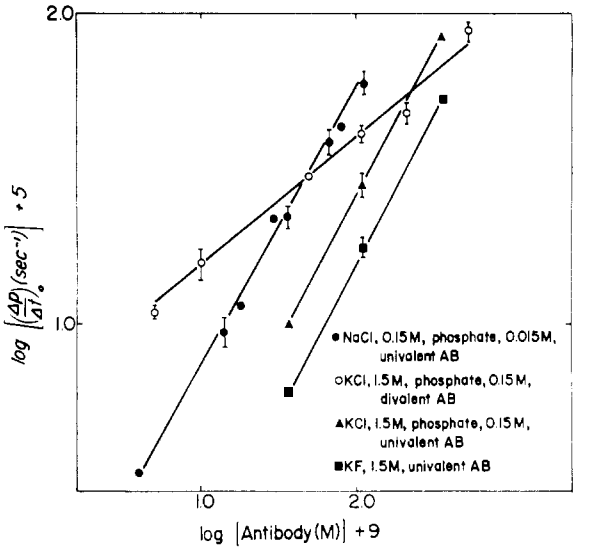

FIGURE 4: Initial rate behavior for the fluorescein-labeled ovalbumin–univalent antiovalbumin system in various ionic media at 1.5 ± 0.5°. All solutions were buffered at pH 7.0. The order of reaction with respect to antibody was determined by means of eq 2: (●) 0.15 M NaCl–0.015 M phosphate–univalent antibody; (▲) 1.5 M KCl–0.15 M phosphate–univalent antibody; (■) 1.5 M KF–0.01 M Tris–univalent antibody; (○) 1.5 M KCl–0.15 M phosphate–divalent antibody.

TABLE I: Order of Reaction as Determined by Initial Rate Measurements in Chaotropic Media[a] (See eq 1 and 2.)

Medium (M)	*AG–AB* System	*AB* Order, N_1	*AG* Order, N_2	Factor of Concn Change (*AB*)	Factor of Concn Change (*AG*)
NaCNS (1.5)	FO[b]–divalent anti O	1.0	1.0	10	10
NaCNS (0.5)	Dansyl bovine serum albumin–divalent anti bovine serum albumin	0.95	1.0	20	20
NaCNS (0.5)	Dansyl bovine serum albumin–univalent anti bovine serum albumin	1.0	1.0	14	20
NaCNS (0.15)	FO–divalent anti O	1.0	1.0	8	16
$NaClO_4$ (1.5)	FO–divalent anti O	1.0	0.95	16	10
$NaClO_4$ (0.15)	FO–univalent anti O	1.0	1.0	5	5
$NaClO_4$ (0.15)	FγG,[c] divalent anti F	1.0	1.0	10	11
NaCl (1.5)	FO–divalent anti O	0.97	1.1	10	10
NaCl (0.5)	Dansyl bovine serum albumin–divalent anti bovine serum albumin	1.0	1.0	10	20
NaCl (0.5)	Dansyl bovine serum albumin–univalent anti bovine serum albumin	0.93	1.0	20	14
NaCl (0.15)	FO–divalent anti O	1.0	1.0	20	10
NaCl (0.15)	Dansyl bovine serum albumin–divalent anti bovine serum albumin	1.0	1.0	20	10
NaCl (0.15)	FγG–divalent anti F	1.0	1.0	10	10
NaCl (0.15)	Dansyl bovine serum albumin–univalent anti bovine serum albumin	1.0	1.0	20	10
NaCl (0.15)	FγG–univalent anti F	1.0	1.0	10	10
KCl (1.5)	FO–divalent anti O	1.0	1.0	10	50
KCl (0.15)	FO–divalent anti O	0.93	1.0	10	10

[a] All experiments were carried out in 0.01 M Tris buffer, pH 7.0, at 1.5 ± 0.5°. [b] FO and anti O denote fluorescein-labeled ovalbumin and antiovalbumin, respectively. [c] FγG and anti F refer to fluorescein-labeled γ-globulin and antifluorescein, respectively.

tropic ions such as chloride, perchlorate, and thiocyanate, a rate law which is first order with respect to antibody and first order with respect to antigen is followed, *i.e.*

$$-\left(\frac{d(AG)}{dt}\right)_{t\to 0} = k'(AB)(AG) \qquad (7)$$

whereas in the presence of such ions as phosphate, sulfate, and fluoride, the rate law is essentially half-order with respect to antibody, but remains first order with respect to antigen, *i.e.*

$$-\left(\frac{d(AG)}{dt}\right)_{t\to 0} = k''(AB)^{1/2}(AG) \qquad (8)$$

Effect of Specific Ions on the Empirical Rate Law for the Antigen–Univalent Antibody (Fab) Reaction. Further initial rate studies were made on the binding of antigen to univalent antibody (Fab). Examples of the determination of N_1 for the ovalbumin–antiovalbumin and for the bovine serum albumin–anti bovine serum albumin systems are shown in Figures 4 and 5, respectively, whereas the determination of N_2 for the bovine serum albumin–anti bovine serum albumin system is shown in Figure 6. A complete tabulation of the values of N_1 and N_2 from other measurements is given in Tables I and II. The results differ from those obtained for the divalent antibody systems in that the reaction rate for the antigen–univalent antibody systems in all the ionic media studied, obeys eq 7; *i.e.*, the rate is first order with respect to antibody and first order with respect to antigen.

Specific Salt Effects on Antigen–Antibody Binding Kinetics as Studied by the Integrated Rate Method. Integrated rate measurements (eq 5) were made under pseudo-first-order conditions both to confirm the results of initial rate measurements and to determine specific salt effects on the back-reaction. Plots of S, $(k_1(AB)_0 + k_{-1})/2.3$, *vs.* antibody concentration are shown in Figure 7 for the fluorescein–labeled ovalbumin–divalent antiovalbumin system. Similar plots for the dansyl bovine serum albumin antigen and its corresponding divalent

TABLE II: Order of Reaction as Determined by Initial Rate Measurements in Nonchaotropic Media[a] (See eq 1 and 2).

Medium (M)	*AG–AB* System	*AB* Order, N_1	*AG* Order, N_2	Factor of Concn Change (*AB*)	Factor of Concn Change (*AG*)
KCl (1.5) K_2HPO_4 (0.1) KH_2PO_4 (0.05)	FO[b]–divalent anti O	0.44	1.0	50	50
	FγG[c]–divalent anti F	0.53	1.0	10	10
	FO–univalent anti O	1.0	1.0	10	5
	FγG–univalent anti F	1.0	1.0	10	7
K_2HPO_4 (0.67) KH_2PO_4 (0.33)	FO–divalent anti O	0.47	1.0	80	20
	Dansyl bovine serum albumin–divalent anti bovine serum albumin	0.45	1.0	40	10
	Dansyl bovine serum albumin–univalent anti bovine serum albumin	1.0	1.0	40	10
KCl (1.5) K_2HPO_4 (0.01) KH_2PO_4 (0.005)	FO–divalent anti O	0.46	1.0	40	10
NaCl (1.5) Na_2HPO_4 (0.01) NaH_2PO_4 (0.005)	FO–divalent anti O	0.5	1.0	10	10
NaCl (0.15) Na_2HPO_4 (0.01)	FO-divalent anti O	0.61	1.0	200	100
	Dansyl bovine serum albumin–divalent anti bovine serum albumin	0.8	1.0	10	13
Na_2H2–PO_4 (0.005)	FO–univalent anti O	0.92	1.0	30	100
KCl[d] (1.5) K_2SO_4 (0.1)	FO–divalent anti O	0.45	1.0	40	20
KF[d] (1.5)	FO–divalent anti O	0.48	0.93	40	10
KF (1.5)	FO–univalent anti O	1.0	1.0	10	5
NaF[d] (0.5)	Dansyl bovine serum albumin–divalent anti bovine serum albumin	0.6	1.0	40	13
NaF[d] (0.5)	Dansyl bovine serum albumin–univalent anti bovine serum albumin	1.0	1.0	20	13

[a] All experiments were carried out at 1.5 ± 0.5°. [b] FO and anti O denote fluorescein-labeled ovalbumin and antiovalbumin, respectively. [c] FγG and anti F refer to fluoresceine-labeled γ-globulin and antifluorescein, respectively. [d] Buffered with 0.01 M Tris at pH 7.0.

and univalent antibody partners are shown in Figures 8 and 9, respectively. The magnitudes of k_1 and k_{-1} determined from these plots as well as the k values obtained by initial rate measurements are listed in Tables III and IV. Results for univalent antiovalbumin are also included in Table III. The values of $p_b - p_f$ as well as Qb/Qf which are necessary for the proper evaluation of initial second-order rate constants by eq 4 were determined by extrapolation of $p_e - p_f$ values to zero values of both $(AG)_0$ and $1/(AB)_0$ (Dandliker *et al.*, 1964).

Magnitudes of Rate and Equilibrium Constants in Various Ionic Media. It is apparent from Tables III and IV that the stability of *AG*–divalent *AB* and *AG*–Fab complexes (as measured by the kinetically determined equilibrium association constant, $K = k_1/k_{-1}$) is markedly influenced by the particular ionic media. For example, the fluorescein-labeled ovalbumin–divalent antiovalbumin reaction in 1.5 M NaCl has a K value six times as great as that in 1.5 M NaSCN (Table III). Likewise, the dansyl bovine serum albumin–Fab association has a K value in 0.5 M NaF which is seven times as great as that in 0.5 M NaSCN. It should be pointed out that the ionic strength also influences the association constant as can be noted in the ovalbumin–divalent antiovalbumin reaction where a tenfold increase in salt concentration in the range 0.15 to 1.5 M decreases K by roughly a factor of 10. It should be also noted that changes in K due to specific ion effects especially at higher ionic strength are attributable much more to changes in k_1, the bimolecular association rate constant, rather than k_{-1}, the unimolecular dissociation rate constant.

Discussion

Relation of Rate Law to the Hofmeister Series. This communication reports on the effects of several anions on the reaction kinetics of three different antigen–antibody systems:

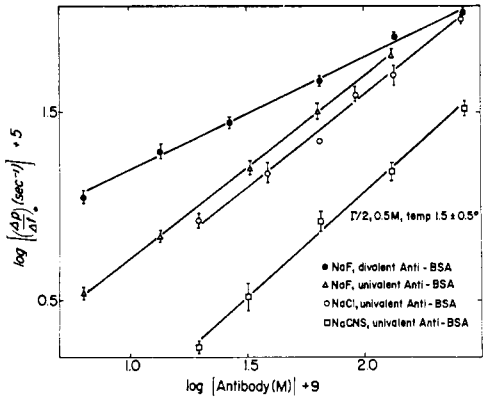

FIGURE 5: Initial rate behavior for the dansyl-labeled bovine serum albumin–univalent anti bovine serum albumin system in various ionic media at 1.5 ± 0.5°; Γ /2 = 0.50. All solutions were buffered at pH 7.0. The order of reaction with respect to antibody concentration was determined by means of eq 2: (●) NaF–divalent antiBSA; (△) NaF–univalent anti bovine serum albumin; (○) NaCl–univalent anti bovine serum albumin; (□) NaCNS–univalent anti bovine serum albumin.

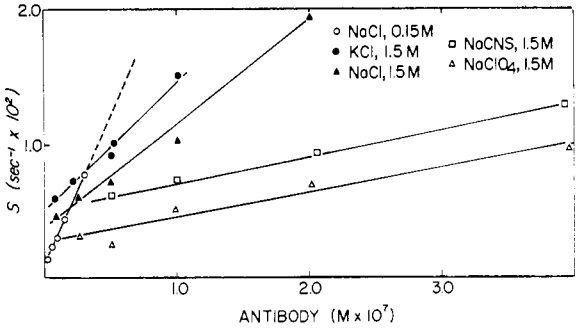

FIGURE 7: Plots of pseudo-first-order rate constants *vs.* divalent antiovalbumin concentration in various ionic media. *S* denotes pseudo-first-order parameter, $k_1(AB) + k_{-1}/2.3$ (see eq 5). The slope and intercept of these plots yield the second-order association constant, k_1, and the first-order dissociation constant, k_{-1}, respectively, for the ovalbumin–antiovalbumin reaction. All experiments were performed in pH 7.0, 0.01 M Tris at 1.5 ± 0.5°. Antigen–antibody preparations were the same as those of Figure 1: (○) 0.15 M NaCl; (●) 1.5 M KCl; (▲) 1.5 M NaCl; (□) 1.5 M NaCNS; (△) 1.5 M $NaClO_4$.

ovalbumin–antiovalbumin, bovine serum albumin–antibovine serum albumin, and fluorescein γG–antifluorescein. These experiments comprise a study of the primary antigen–antibody combination by the technique of fluorescence polarization kinetics. All three antigen–divalent antibody systems have been found to obey a simple second-order rate law in ionic media composed of such chaotropic ions as chloride, perchlorate, and thiocyanate, *i.e.*, $-(d(AG)/dt)_0 = k'(AB)(AG)$, whereas, in the presence of such nonchaotropic ions as phosphate, sulfate, and fluoride, the rate law is more complicated and involves a fractional order with respect to divalent *AB*, *i.e.*,

$$-\left(\frac{d(AG)}{dt}\right)_0 = k''(AB)^{N_1}(AG)$$

where N_1 is usually close to 0.5. Furthermore, since the primary combination between antigen and its corresponding univalent antibody partner, Fab, obeys simple second-order kinetics in all ionic media investigated, intact antibody and Fab antibody must react with antigen by different processes in non-

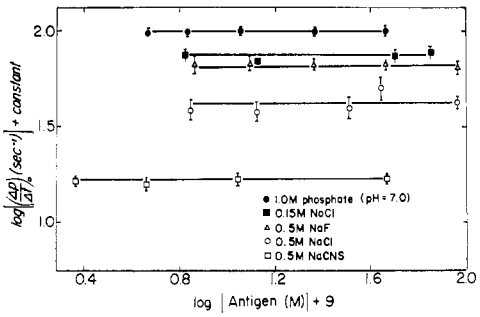

FIGURE 6: Determination of order with respect to antigen for the dansyl-labeled bovine serum albumin–univalent anti bovine serum albumin system in various ionic media at 1.5 ± 0.5° (see eq 1). All solutions were buffered at pH 7.0: (●) 1.0 M phosphate; (■) 0.15 M NaCl; (△) 0.5 M NaCl; (○) 0.5 M NaCl; (□) 0.5 M NaCNS.

chaotropic media. Finally there is a correlation with the magnitude of all the second-order rate constants determined in this study with the Hofmeister series. That is, the value of the second-order rate constant in various media increases according to the following sequence of ions: $SCN^- < ClO_4^- < Cl^- < F^- < SO_4^{2-} <$ phosphate.

These specific anion effects on the kinetics of antigen–antibody binding follow similar trends established in studies involving macromolecular stability (von Hippel and Wong, 1964), enzymatic steady-state reactions (Warren *et al.*, 1966; Warren and Cheatum, 1966), equilibrium dialysis of the hapten–anti-*p*-azobenzoate complex (Pressman *et al.*, 1961), and the dissociation of antigen–antibody complexes (Dandliker *et al.*, 1967). Generally speaking the chaotropic anions such as

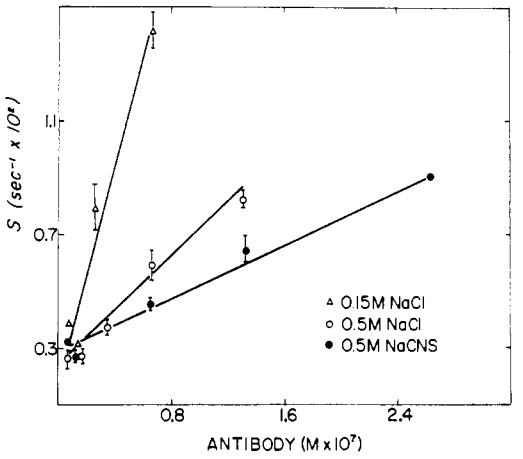

FIGURE 8: Salt effects on reaction kinetics of the dansyl-labeled bovine serum albumin–divalent anti bovine serum albumin system at 1.5 ± 0.5°. *S* denotes pseudo-first-order parameter, $k_1(AB) + k_{-1}/2.3$ (see eq 5). The slope and intercept of these plots yield the second-order association constant, k_1, and the first-order dissociation constant, k_{-1}, respectively. All experiments were performed in pH 7.0, 0.01 M Tris buffer: (△) 0.15 M NaCl; (○) 0.5 M NaCl; (●) 0.5 M NaCNS.

TABLE III: Summary of Rate and Equilibrium Constants for the Fluorescein-Labeled Ovalbumin–Antiovalbumin and the Fluorescein-Labeled γ-Globulin–Antifluorescein Systems in Various Ionic Media.[a]

Medium (M)	k_1 (M^{-1} sec^{-1}) × 10^{-4} [b]	k_{-1} (sec^{-1}) × 10^{2} [b]	K (M^{-1}) × 10^{-6} [c]	Initial Rate Constant k [d] (M^{-1} sec^{-1}) × 10^{-4}
		FO[e]–divalent anti O		
NaCNS[f] (3.0)				10^{-2}–10^{-1}
NaCNS (1.5)	4.0	1.2–1.0	3.3–4.0	3.8 ± 0.4
$NaClO_4$ (1.5)	3.7	0.5–1.0	3.7–7.4	2.8 ± 0.2
NaCl (1.5)	18	0.8	23	15 ± 3
KCl (1.5)	23	1.1	21	24 ± 2
NaCNS (0.15)	29	0.5–1.0	30–60	20 ± 2
NaCl (0.15)	48	0.2	240	50 ± 6
KCl (0.15)				68 ± 8
		FO, univalent anti O		
NaCl (0.15), Na_2HPO_3 (0.01), NaH_2PO_4 (0.005)	20	0.8	25	15 ± 3
KF (1.5)	12	0.5	24	
$NaClO_4$ (0.15)	11	0.5	22	10 ± 3
KCl (1.5), K_2HPO_4 (0.1), KH_2PO_4 (0.05)	8	0.7	1.1	8 ± 1
		FγG,[g] divalent anti F		
NaCl (0.15)				62 ± 4
		FγG, univalent anti F		
KCl (1.5), K_2HPO_4 (0.1), KH_2PO_4 (0.05)				3 ± 0.2
NaCl (0.15)				15 ± 1

[a] All experiments were carried out at 1.5 ± 0.5° in solutions buffered at pH 7.0. [b] Defined by eq 6. [c] $K = k_1/k_{-1}$. [d] Defined by eq 4. Typical $p_b - p_f$ and Qf/Qb values used to evaluate k for the anti O systems were 0.05 and 1.0, respectively. Values of $p_b - p_f$ and Qf/Qb used to evaluate k for the divalent anti F systems were 0.13 and 1.8, respectively, while $p_b - p_f$ and Qf/Qb values used for the univalent anti F systems were 0.14 and 2.9, respectively. [e] FO and anti O denote fluorescein-labeled ovalbumin and antiovalbumin, respectively. [f] All solutions except those which are explicitly listed as containing phosphate buffer were buffered with 0.01 M Tris. [g] FγG and anti F refer to fluorescein-labeled γ-globulin and antifluorescein, respectively.

SCN^-, ClO_4^-, and Cl^- which are at one end of the Hofmeister series, tend to unfold and dissociate macromolecules whereas, the anions which are at the other end (phosphate, sulfate, and fluoride), tend to promote folding and association. It is of particular interest that these effects extend even to the realm of small molecule interactions since they have been observed in the study of salt effects on SN2 reactions between 2,4-dinitrochlorobenzene with aniline, hydroxide, or thiophenoxide (Bunton and Robinson, 1968). The correlation of our kinetic parameters with the Hofmeister series is not readily explained on the basis of direct electrostatic, anion (competitive) binding to a positively charged active site on the antibody molecule since the nonchaotropic anions actually enhance the *AB–AG* combination rather than impede it as one might expect if indeed direct anion binding occurred. Similarly, it is equally difficult to interpret these effects in terms of anions competitively binding to the net negatively charged antigen molecule, since it is evident that whatever anion binding does occur it is quite different for the three antigens studied (Klotz, 1953) and yet the same salt trends prevail no matter which antigen is selected. However an explanation of these salt effects on antigen–antibody kinetics can be made in terms of the solvation and conformational changes which occur when antigen and antibody molecules combine and we will now develop this interpretation.

Plausible Reaction Mechanisms

The simplest mechanism consistent with the observed facts that antigen–divalent antibody reactions in chaotropic media, and antigen–Fab reactions in all ionic media obey second-order kinetics in the initial stages of reaction is one which involves a bimolecular combination between antigen and antibody:

$$AB + AG \xrightarrow{k_1} AB\text{–}AG \qquad (9)$$

TABLE IV: Summary of Rate and Equilibrium Constants for the Dansyl-Labeled Bovine Serum Albumin–Anti Bovine Serum Albumin System in Various Ionic Media.[a]

Medium (M)	k_1 (M^{-1} sec^{-1}) $\times 10^{-4}$ [b]	k_{-1} (sec^{-1}) $\times 10^{2}$ [b]	K (M^{-1}) $\times 10^{-6}$ [c]	Initial Rate, k (M^{-1} sec^{-1}) $\times 10^{-4}$ [d]
	Dansyl bovine serum albumin–divalent anti bovine serum albumin			
NaCl[e] (0.15)	34	0.2	170	32 ± 4
NaCl (0.5)	11	0.7	16	11 ± 2
NaCNS (0.5)	7.9	0.6	13	6.8 ± 2
	Dansyl bovine serum albumin–univalent anti bovine serum albumin			
NaCl (0.15)	11	1.0	11	10 ± 1.2
K_2HPO_4 (0.67) KH_2PO_4 (0.35)	10	0.5	20	11 ± 1.5
NaCl (0.5)	7.3	0.7	10	7.5 ± 1.0
NaCNS (0.5)	2.0	0.6	3	1.9 ± 0.2
NaF (0.5)	13.0	0.6	21	10 ± 1.0

[a] All studies were carried out at 1.5 ± 0.5° in solutions buffered at pH 7.0. [b] Defined by eq 5. [c] $K = k_1/k_{-1}$. [d] Defined by eq 4. Typical values of $p_b - p_f$ and Qf/Qb used to evaluate k were 0.06 and 1.0, respectively. [e] All solutions except those which are explicitly listed as containing phosphate were buffered with 0.01 M Tris.

The fact that the magnitudes of the second-order rate constants are particularly sensitive to the nature of the specific anion present implies that the bimolecular process involved in eq 9 is not diffusion controlled but involves important structural changes. A recent determination of an activation energy of 12 kcal/mole for the ovalbumin–divalent antiovalbumin combination (Levison *et al.*, 1968) as well as interpretations of electromicrographs (Feinstein and Rowe, 1965; Valentine and Green, 1967) support this viewpoint. It has been suggested that the divalent antibody molecule during its combination with its hapten or antigen partner clicks open around a central hinge and acquires an open rod-like shape, as compared to its more globular shape prior to reaction (Feinstein and Rowe, 1965). It is our view that such conformational changes probably involve solvent reorganization and solvent loss, and/or unfolding during formation of the activated complex. The idea of solvent loss is suggested by the fact that estimations of the entropy of activation for the ovalbumin–divalent antiovalbumin reaction in 0.15 M NaCl at pH 7.0 yields an unusual, high positive value (Levison *et al.*, 1968) when compared with other bimolecular associations (Laidler, 1965) as well as to the hapten–antihapten reaction (Day *et al.*, 1963). Furthermore if the activated complex is less solvated than the isolated reactants, then anions which compete more effectively for solvent molecules will tend to promote the reaction rate. This is precisely what is observed experimentally. Anions, with high charge density, tend to enhance the reaction rate whereas anions with low charge density tend to depress the reaction. The same solvation effect seems to be also important in many organic SN2 reactions in protic and aprotic solvents (Parker, 1967), where reorganization of solvent molecules oriented around both polar and apolar groups presumably occurs in the formation of the transition state. These dehydration effects are probably important not only in formation of the activated complex but also of the final product. This can be concluded from the fact that similar anion effects on the equilibrium binding of hapten to anti-*p*-azobenzoate antibody have been observed (Pressman *et al.*, 1961). Furthermore, cations have been reported to increase the equilibrium binding of hapten to anti-azophenyltrimethylammonium antibody also in order of increasing charge density (Grossberg *et al.*, 1962).

To account for the fractional order observed for antigen–divalent antibody kinetics in nonchaotropic media, we have proposed a mechanism in which there is rapid reversible forma-

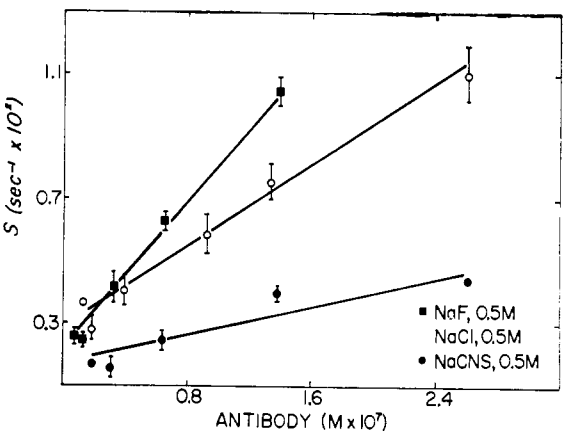

FIGURE 9: Specific ion effects on the reaction kinetics of the dansyl-labeled bovine serum albumin–univalent anti bovine serum albumin system at 1.5 ± 0.5 °; $\Gamma/2 = 0.50$. S denotes pseudo-first-order parameter, $k_1(AB) + k_{-1}/2.3$ (see eq 5). The slope and intercept of these plots yield the second-order association constant, k_1, and the first-order dissociation constant, k_{-1}. All experiments were performed in 0.01 M pH 7.0 Tris buffer: (■) NaF; (○) NaCl; (●) NaCNS.

tion of a loosely held encounter pair of antigen and antibody macromolecules (Levison and Dandliker, 1969). A slower, unimolecular process in which the final antigen–antibody complex is formed, then follows

$$AB + AG \underset{}{\overset{\text{fast}}{\rightleftharpoons}} AB\text{---}AG \qquad (10)$$

$$AB\text{---}AG \xrightarrow[\text{slow}]{k_p} AB\text{–}AG \qquad (11)$$

It is also assumed that the reacting sites exhibit a nonuniformity which is manifested in the encounter pair formation and that the encounter pair formation equilibrium involves a Sips distribution of binding energies (Sips, 1948). The initial rapid interaction between antigen and antibody may be thought of as relatively weak and occurring at a few contact points (possibly near or at one of the antibody specific sites) in which hydrophobic and or electrostatic bonding occur. Many solvent molecules would still be interdispersed between the antigen and antibody molecules which make up the loose encounter pair. The rate-determining step yielding the final product would involve a gradual realignment of antigen and antibody bonds in which the second valence of the antibody molecule may play an important role and in which solvent molecules may be released either as the result of neutralization of charge and/or of hydrophobic bonding.

The proposed macromolecular encounter pair in many ways resembles the ion pair whose chemical behavior is dependent on whether the reactants are in intimate contact or solvent separated. The ion-pair concept has been used extensively in studies concerning free-radical behavior (Szwarc, 1968), organic solvolysis mechanisms (Winstein *et al.*, 1965), and inorganic complexation reactions (Eigen, 1960; Eigen and Tamm, 1962). In fact, the above described encounter pair mechanism is quite similar to the general mechanism of complexation of ligand to metal ions which involves formation of an ion pair followed by a rate-determining dissociation of one or more water molecules (Eigen, 1960; Eigen and Tamm, 1962). However, it should be pointed out that the initial encounter pair formation unlike ion-pair reactions is not necessarily diffusion controlled. Furthermore the macromolecular encounter pair stability depends not only on electrostatic forces as in ion pairing but also on apolar interactions.

The second-order rate constants obtained for divalent antibody systems in chaotropic media suggest that in chaotropic media only one of the antibody valences may be kinetically important and that the rearrangement of the encounter pair (eq 11) occurs very rapidly compared with the equilibrium involving encounter pair formation (eq 10). This idea is supported by the fact that Fab (univalent) fragments in all media obey second-order kinetics and they behave hydrodynamically as simple globular proteins as opposed to the much more flexible divalent form (Noelken *et al.*, 1965). One would also expect that the divalent form with its high degree of flexibility should be much more sensitive to intramolecular folding effects (caused by the nonchaotropic high charge density anions) than the more globular univalent form. It may be that intramolecular folding is in part responsible for the nonuniformity of sites in formation of the encounter pair. In these terms the kinetic effects of intramolecular folding of the antibody molecule can be minimized either by splitting whole antibody into Fab fragments or by changing the environment from a nonchaotropic to a chaotropic medium where hydrophobic bonds are broken. Finally, the encounter pair mechanism can also be thought of in terms of a model previously presented to explain fractional-order rate laws which were observed in heterogeneous catalysis (Halsey, 1949). The key idea is that for systems which have a nonuniformity of binding sites having a complete available range of activation energies, there is no single rate-determining step but rather different rate-determining steps on different types of sites. On some sites, encounter pair formation (eq 10) would be rate limiting whereas on others, rearrangement of the encounter pair (eq 11) would be rate limiting. On a third set both processes would be equally slow. It is assumed that the sum of the two activation energies for any site is always equal to a constant overall activation energy. This last statement implies that on one kind of site, slow encounter pair formation (high activation energy), would lead to rapid rearrangement (low activation energy), and rapid encounter pair formation (low activation energy) would necessitate a slow rearrangement (high activation energy).

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Characterization of Antibodies of High Affinity and Specificity for the Digitalis Glycoside Digoxin*

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ABSTRACT: Immunization of rabbits with a conjugate of the steroid glycoside digoxin coupled by a periodate oxidation method to amino groups of lysine in human serum albumin resulted in the production of high titer digoxin-specific antibodies with exceptionally high affinity and specificity late in the immune response. Studies of antisera from an animal heavily immunized over a period of 97 weeks showed 5.8 mg/ml of digoxin-specific antibody. The average intrinsic affinity constant (K_0) of this antiserum, determined by Sips analysis of equilibrium dialysis data, was 1.7×10^{10} M^{-1}; relative homogeneity was indicated by a heterogeneity index (a) of 0.92. The K_0 for digitoxin, which differs by only a single OH group at the C_{12} position, was 32-fold lower. A ninefold increase was found in K_0 for digoxin between the 6th and 32nd weeks of immunization in another animal serially studied. Hapten inhibition experiments with digitoxin deslanoside, digoxigenin, cholesterol, cortisol, dehydroepiandrosterone, 17β-estradiol, progesterone, and testosterone were carried out on a number of antisera utilizing a dextran-coated charcoal method for separation of bound and free ligand.

High specificity for antigenic determinants of the steroid nucleus of digoxin, tending to increase with time following immunization, was demonstrated. These antibodies may be employed in the measurement of 3×10^{-13} mole/ml concentrations of digoxin in physiologic fluids.

Previous studies have shown that antibodies specific for steroid haptens can be obtained by immunization of animals with antigens consisting of steroid molecules coupled to carrier proteins (Lieberman *et al.*, 1959; Zimmering *et al.*, 1967; Gross *et al.*, 1968). It has been suggested that such antibodies might be useful in the measurement of low concentrations of steroid compounds (Goodfriend and Sehon, 1961). This principle has been applied to the determination of 17β-estradiol and testosterone (Beiser and Erlanger, 1967) and the steroid glycosides digoxin (Butler and Chen, 1967) and digitoxin (Oliver *et al.*, 1968). The steroid hapten–antibody interaction has also been advanced as a possible model for hormone binding by target tissue receptors (Zimmering *et al.*, 1967).

Previous studies of average intrinsic affinity constants (K_0) for steroid-specific antibodies, obtained by immunization of unbred ewes with steroid–bovine serum albumin conjugates (Zimmering *et al.*, 1967), yielded values of the order of 10^5 M^{-1}. These appear too low to allow the practical use of such antibodies in radioimmunoassay systems where sensitivities of the order of 10^{-12} mole are required. The specificity of antibodies to steroid haptenic determinants has been evaluated by quantitative precipitin analysis, equilibrium dialysis, and hapten inhibition (Lieberman *et al.*, 1959; Zimmering *et al.*, 1967; Gross *et al.*, 1968). These studies have generally shown substantial cross-reactivity among related steroid compounds when tested against steroid-specific antisera.

Butler and Chen (1967) have reported a technique for coupling the steroid glycoside digoxin to ϵ-amino groups of

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