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Kinetics of Neutralization of Bacteriophage f2 by Rabbit γG-Antibodies*

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Abstract. The neutralization of bacteriophage f2 by intact γG-immunoglobulin or fragments is first order with respect to both bacteriophage and antibody. Minimum values for the rate constants are of the order of $10^7\ M^{-1}\ \text{sec}^{-1}$. The temperature dependence of the rates corresponds to the activation parameters: $\Delta H\ddagger = 6.7$ kcal mole^{-1} and $\Delta S\ddagger = -4$ cal deg^{-1} mole^{-1} (γG-immunoglobulin); $\Delta H\ddagger = 8.0$ kcal mole^{-1} and $\Delta S\ddagger = -0.9$ cal deg^{-1} mole^{-1} (5*S* pepsin fragment); and $\Delta H\ddagger = 13.3$ kcal mole^{-1} and $\Delta S\ddagger = 12$ cal deg^{-1} mole^{-1} (3.5*S* fragment). The kinetic observations are consistent with the view that the binding of a single antibody molecule can bring about phage neutralization. There are two ways in which a single antibody molecule can affect neutralization: (1) binding at or near some critical site on the phage may block its function, (2) binding may disturb the general architectural design of the protein shell of the phage. Although the rate of neutralization varied directly with antibody size, consideration of the activation parameters speaks against the dependence of neutralization on simple steric factors. Addition of antibodies directed against rabbit γG-immunoglobulin or the 5*S* pepsin fragment caused approximately a threefold neutralization enhancement. This enhancement may result from the detection of a class of infectious bacteriophage antibody complexes.

Introduction. The bacteriophage f2 is a small "spherical" particle[1] in which the major coat component is approximately 180 identical protein molecules.[2, 3] This particular virus was chosen for our investigation into the mechanism of bacteriophage neutralization because of the simplicity of its structure as compared to many other bacteriophage.

It is generally believed that steric factors are of major importance in the neutralization of bacteriophage by specific antiserum. The evidence supporting this view has been obtained by inference from studies of the kinetics of neutralization of bacteriophage, but is by no means conclusive.[4–8]

In view of this we have undertaken a kinetic study of the mechanism of neutralization of bacteriophage f2 by rabbit antibodies. The temperature dependence of the kinetics of neutralization and the effects of antiserum and its fragments on neutralization have been used in this study.

Methods. Antigen: The bacteriophage f2 was grown and purified as described by others.[1] Five mg of the virus were emulsified in 10 ml of Freund's complete adjuvant (Difco). Hyperimmunization of young New Zealand white rabbits was carried out

by weekly footpad or subcutaneous injections of 0.5 ml of the antigen preparation until a higher titer antiserum was obtained, at which time the rabbits were sacrificed and the serum obtained from clotted blood was stored at −10°C. Rabbit γG-immunoglobulins and immunoglobulin fragments were similarly prepared as antigens and appropriate antiserum was obtained by immunization of adult opossums (*Didelphis virginiana*).

Preparation of γG-immunoglobulin and its fragments: The γG-immunoglobulin was isolated from rabbit serum by anionic exchange chromatography.[9] The γG-immunoglobulin was batch eluted from the column with 0.01 *M* sodium phosphate buffer (pH 7.2).

The pepsin fragment was prepared from γG-immunoglobulin essentially as described by Nisonoff *et al.*[10] Digestion with pepsin (Sigma Chemical Co.) was carried out in 0.01 *M* sodium acetate buffer (pH 4.0) at 37°C for 16 hr. The product of digestion was purified by gel filtration on Sephadex G-25 equilibrated with 0.1 *M* Tris buffer (pH 8.0) in 0.15 *M* NaCl. The 3.5*S* fragment was prepared by reduction and alkylation of the 5*S* pepsin fragment.[7]

The γG-immunoglobulin and its fragments were partially characterized by immunoelectrophoresis[11] and estimations of their sedimentation coefficients were obtained using a Spinco model E ultracentrifuge as previously described.[7] The protein concentration of these preparations was estimated from their optical density at 280 mμ using an extinction coefficient of 1.4 optical density units/mg protein/ml. Calculations of the molar concentration were based on molecular weights of 150,000, 100,000, and 50,000 for γG-immunoglobulin, the 5*S* pepsin fragment, and the 3.5*S* fragment, respectively.

Kinetic techniques: The kinetics of virus neutralization was followed by measuring the number of plaque-forming units (*P*) using the agar overlay technique[12] with *Escherichia coli* K-37 as the bacterial host and the bacteriophage f2.[7] The reaction mixture consisted of tryptone broth (pH 7.0) containing 10 gm/l tryptone; 8 gm/l NaCl; 1 gm/l glucose; 1 gm/l yeast extract; and 220 mg/l $CaCl_2$. Customarily the virus was incubated for 50 min at 37°C before the reactions were begun. The virus was incubated for an additional 10 min at the reaction temperature.

Anti-antibody reactions were begun by preparing a series of four reaction mixtures which were identical with respect to their content of bacteriophage and antibody. These reaction mixtures were incubated at 37°C. One of the reaction mixtures was used for direct neutralization and contained no anti-antibody. Anti-antibody was added to the other reaction mixtures at 10, 20, or 30 min during neutralization. Aliquots were then removed from the reaction mixtures containing anti-antibody at intervals for plating.

Results. Bacteriophage neutralization: In the presence of a large excess of γG-immunoglobulin or its fragments, bacteriophage f2 neutralization exhibits apparent first-order kinetics. The results of representative experiments using the γG-immunoglobulin, its 5*S* pepsin, and the 3.5*S* fragment are illustrated in Figures 1 and 2. Variation of the antibody concentration demonstrated that, in the region of antibody excess, the neutralization rate is proportional to the antibody concentration. Therefore, the reaction is first order with respect to antibody and first order with respect to bacteriophage.

Second-order rate constants, k, could then be calculated from the slope of the first-order semilogarithmic plots illustrated in Figures 1 and 2 and the initial concentration of anti-f2 immunoglobulin or its fragments. Average k values obtained from multiple studies at 37°C are $2.01 \pm 0.20 \times 10^7\ M^{-1}\ \text{sec}^{-1}$, $0.95 \pm 0.05 \times 10^7\ M^{-1}\ \text{sec}^{-1}$ and $0.15 \pm 0.04 \times 10^7\ M^{-1}\ \text{sec}^{-1}$ for γG-immunoglobulin, the 5*S* pepsin fragment and 3.5*S* fragment respectively (Table 1).

The effect of temperature on the neutralization constants is illustrated in Figure 3. Apparent activation energies (E_a) calculated from the Arrhenius

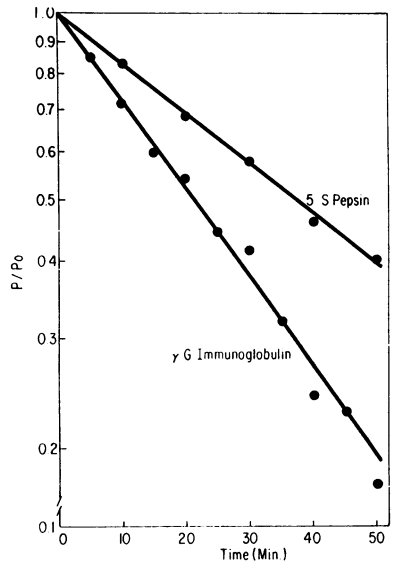


FIG. 1.—Neutralization of bacteriophage f2 by rabbit γG-immunoglobulin (initial concentration 3.7×10^{-6} mg/ml) and its 5-S pepsin fragment (initial concentration 3.3×10^{-6} mg/ml) at 37°C, pH 7.0, in tryptone broth as described in *Methods*. P/P_0 = ratio of plaque-forming units at time t and time 0.

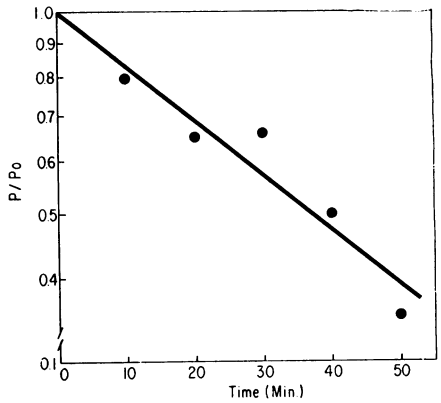


FIG. 2.—Neutralization of bacteriophage f2 by the 3.5*S* fragment of rabbit γG-immunoglobulin (initial concentration 1.3×10^{-5} mg/ml). Conditions as in Figure 1.

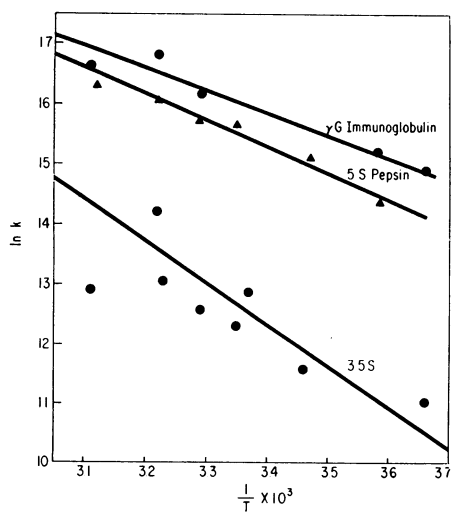


FIG. 3.—Arrhenius plots for γG-immunoglobulin, 5*S* pepsin fragment, and 3.5*S* fragment. T = absolute temperature; k is the second-order rate constant (M^{-1} sec^{-1}) calculated from the slope of the first-order plots as illustrated in Figures 1 and 2 and the initial concentration of anti-f2 immunoglobulin or its fragments.

plots are 7.3, 8.6, and 13.9 kcal $mole^{-1}$ for the intact immunoglobulin, the 5*S* fragment, and the 3.5*S* fragment, respectively. Calculations of apparent enthalpy and entropy of activation were made from the activation energy and rate

TABLE 1. *Comparison of neutralization by antibody preparations.**

	γG-immuno-globulin	5S pepsin fragment	3.5S fragment
k (M^{-1} sec^{-1} × 10^7)	2.01 ± 0.20	0.95 ± 0.05	0.15 ± 0.04
Relative activity (%)	100	47	7
E_a (kcal $mole^{-1}$)	7.3	8.6	13.9
$\Delta G‡$ (kcal $mole^{-1}$)	7.8	8.3	9.4
$\Delta H‡$ (kcal $mole^{-1}$)	6.7	8.0	13.3
$\Delta S‡$ (cal deg^{-1} $mole^{-1}$)	−4	−0.9	12

* k, $\Delta G‡$, $\Delta H‡$, $\Delta S‡$ refer to values at 37°C; standard error included for values of k. k and $\Delta S‡$ are minimal values while $\Delta G‡$ is a maximum value as discussed in the text.

data. Values are included in Table 1. It should be emphasized that the calculations are based on the molar concentration of γG-immunoglobulin or fragments rather than on the specific antibody concentrations, and so the reported second-order rate constants and apparent activation entropies represent minimum values while the activation free energy represents a maximum value.

Neutralization enhancement by anti-immunoglobulin serum: The addition of excess opossum anti-rabbit γG-immunoglobulin serum to a phage-antiphage reaction mixture markedly altered the kinetics of neutralization. Figure 4 shows the results of a representative experiment in which anti-γG-immunoglobulin serum was added to aliquots of the phage-antiphage reaction mixture at various times after the start of the reaction. Bacteriophage infectivity dropped to a steady value rapidly after the addition of the anti-antibody.

Plots of the logarithm of the final phage survival levels (Fig. 4) as a function of the time of addition of anti-antibody were linear. An "amplified neutralization constant," k_A, was then calculated from the slope of these semilogarithmic plots and the initial concentration of anti-f2 immunoglobulin. The "amplified

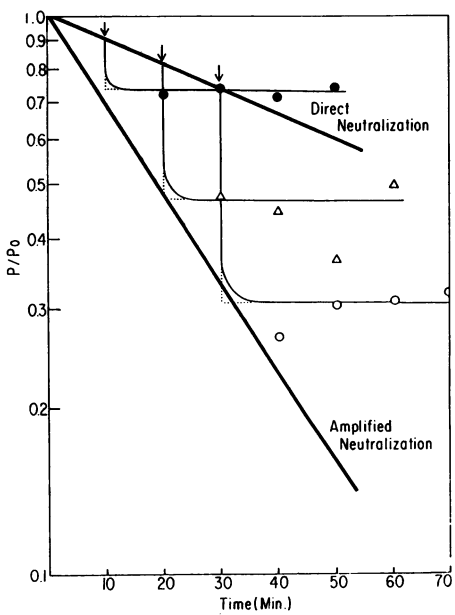


FIG. 4.—Enhancement of neutralization by γG-immunoglobulin with excess opossum anti-rabbit γG. Direct neutralization was carried out with rabbit γG-immunoglobulin (initial concentration 1.6 × 10^{-6} mg/ml). The arrows indicate the points at which opossum anti-rabbit serum was added to aliquots of reaction mixtures. Amplified neutralization determined as described in *Methods*.

neutralization constant" in Figure 4 is $5.7 \times 10^7\ M^{-1}\ \text{sec}^{-1}$. Calculation of the ratio of the "amplified neutralization constant" and the normal neutralization constant (k_N) demonstrates an approximate threefold enhancement of viral neutralization ($k_A/k_N \cong 3$). Average values are indicated in Table 2.

Studies using the 5*S* pepsin fragment and the 3.5*S* fragment gave results similar to those obtained with γG-immunoglobulin. Calculation of average k_A yielded $2.2 \times 10^7\ M^{-1}\ \text{sec}^{-1}$ and $0.53 \times 10^7\ M^{-1}\ \text{sec}^{-1}$ for the 5*S* pepsin fragment and the 3.5*S* fragment, respectively. Determinations of the k_A/k_N ratios showed that amplification was approximately threefold in each case ($k_A/k_N \cong 3$). Table 2 summarizes the results of these studies.

The results obtained with antibodies directed against the 5*S* fragment gave results similar to those obtained with antibodies directed against intact immunoglobulins. In each case the amplification was approximately threefold. Table 2 summarizes the results.

TABLE 2. *Amplification on addition of excess opossum anti-rabbit serum.*

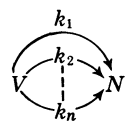
	Anti γG		Anti 5*S* pepsin	
Antibody	$k_A(M^{-1}\ \text{sec}^{-1})$	Amplification	$k_A(M^{-1}\ \text{sec}^{-1})$	Amplification
γG	5.5×10^7	2.7	6.1×10^7	3.0
5*S* pepsin	2.2×10^7	2.3	3.3×10^7	3.4
3.5*S*	0.53×10^7	3.5	0.48×10^7	3.2

k_N = normal neutralization constant.
k_A = amplified neutralization constant.
k_A/k_N = amplification.

In order to further investigate the mechanisms of the anti-antibody amplification, antibacteriophage preparations were preincubated with anti-antibodies. In every case, the phage neutralizing activity was removed. This suggests that antibody complexes which are formed can no longer neutralize the bacteriophage. It implies that in the amplification studies all remaining free antibacteriophage activity is removed by the anti-antibody.

Discussion. The observation that f2 neutralization in the presence of an excess of γG-antibodies is first order in bacteriophage and exhibits no lag phase is consistent with a view that neutralization results from the binding of a single antibody molecule. This observation does not rule out the possibility that there is a class of neutralizing sites, in which the occupation of any *one* site results in neutralization.

A mechanism in which neutralization results from the binding of a single antibody molecule to a specific neutralizing site or class of neutralizing sites can be represented by the formulation:



(1)

where V represents an active bacteriophage and N represents a bacteriophage which has been neutralized by binding antibody at a neutralizing site. The k_1,

$k_2 - - - k_n$ represent first-order rate constants for neutralization. Because the antibody is present in large excess, its concentration is essentially constant and therefore not expressed explicitly in this formulation.

In accordance with this formulation, the rate of disappearance of active bacteriophage is:

$$-d[V]/dt = (k_1 + k_2 + \ldots k_n)[V] = k_N[V]$$

where $[V]$ is the concentration of active phage, n is the number of neutralizing sites on the phage, and $k_N = \sum_1^n k_i$.

The observed kinetics are, of course, consistent with other possible mechanisms of neutralization, but they clearly do not support a simple mechanism requiring the binding of more than one antibody for neutralization. Such a mechanism would lead to an initial lag in the neutralization[13-16] except in the special case where binding to one of the required neutralizing sites is much slower than the rest.

The observed inactivation rates are close to the maximum rate which would be observed if every encounter between antibody and phage resulted in inactivation. In the absence of charge effects, it would be predicted that diffusion would limit the second-order rate constant to about $10^8\ M^{-1}\ \text{sec}^{-1}$.[17, 18] The calculation assumes that: (*a*) the sum of diffusion coefficients for a phage particle and an antibody molecule is approximately $5 \times 10^7\ \text{cm}^2\ \text{sec}^{-1}$, (*b*) the antibody molecule can approach the phage binding site from only one side, and (*c*) the reaction distance is approximately 10 Å. The observed apparent activation energies of 7.3, 8.6, and 13.9 kcal mole^{-1} for the intact antibody, the 5*S* pepsin fragment, and the 3.5*S* fragment, respectively, are greater, however, than the apparent activation energy for a diffusion controlled reaction (4 to 5 kcal mole^{-1}).

It has been observed that the rates at which intact immunoglobulin or its fragments cause bacteriophage neutralization vary with their size.[5, 6] From this it has been concluded that the differences in rate are a simple reflection of steric factors. The view is that because the intact antibody is larger than the fragments it is more likely to cause neutralization by sterically blocking a site necessary for bacteriophage infectivity.

Comparison of the activation parameters $\Delta H\ddagger$ and $\Delta S\ddagger$ obtained from the temperature dependence of the neutralization rates in experiments using γG-immunoglobulin, its 5*S* pepsin, and the 3.5*S* fragment suggest that at least in the case of the bacteriophage f2 neutralization the conclusions are unwarranted. The differences in rate do not appear to be simply related to molecular size and the probability of blocking a critical site.

In all cases relatively large favorable changes in apparent activation enthalpies are accompanied by almost equally large unfavorable changes in apparent activation entropies. The observed trend in rate constants for neutralization by the 3.5*S* fragment, 5*S* fragment, and intact antibody is very small compared to the large, but nearly compensating changes in the contributions due to $\Delta H\ddagger$ and $\Delta S\ddagger$. More importantly, the observed value of $\Delta S\ddagger$ decreases with increasing size of the neutralizing molecule ($\Delta S\ddagger$ for γG-immunoglobulin $<5S$ pepsin

fragment <3.5S fragment). This is exactly opposite to what would have been anticipated were neutralization to depend on simple steric factors.

Neutralization enhancement by anti-immunoglobulin serum may be the result of binding an anti-antibody molecule to an active bacteriophage-antibody complex. In a sense amplified neutralization may represent an assay for a class of infectious bacteriophage-antibody complexes, in which case it would be predicted that the observed amplification is due almost entirely to an increase in the value of $\Delta S\ddagger$. However, since the amplification is only threefold the expected increase in $\Delta S\ddagger$ would be too small to measure.

There are two ways in which the binding of a single antibody molecule can affect neutralization: (1) binding at or near some critical site on the phage may block its function, (2) binding may disturb the general architectural design of the protein shell of the phage. The first possibility implies that there is one patch of the shell which is functionally important and is different from the rest of the shell.[3] The second possibility is in accord with the proposals that small viruses are constructed from identical structural subunits arranged in a regular manner.[19–22] A number of "spherical" viruses for example contain subunits arranged in a surface lattice with icosahedral symmetry.[23] The binding of an antibody molecule to certain antigenic sites on a subunit may cause changes in its shape or orientation within the surface lattice. The maintenance of intersubunit binding contacts may require that structural changes in one subunit induce corresponding changes in its neighbors; thus, the effect of binding of a single antibody molecule can be propagated over the virus particle.

Neutralization enhancement by anti-immunoglobulin serum can be accounted for in this model by supposing that antibody binding to some of the antigenic sites is not sufficient to cause direct neutralization, but anti-antibody binding to an antibody already attached to such a site disturbs the structure of the protein shell sufficiently to cause neutralization.

Alternatively, in terms of the possibility of a critical site, steric enhancement of neutralization by anti-antibody molecules may result from their binding to antibacteriophage molecules near to but not blocking this critical site.

In either case if the rate constants for antibacteriophage binding to the various antigenic sites are essentially the same, then the amplification is a measure of the relative numbers of the two classes of neutralizing sites, amplified and direct. This could account for the similarity of amplification when γG-antibody, 5S pepsin fragments, and 3.5S fragments are used.

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