ear and pass through the origin, and the slopes increase with increasing temperature. Nevertheless, the possibility that a charge-transfer complex is present in these solutions is not fully eliminated. Even the increase in absorption with increasing temperature is possible depending upon the extent and energies of solvation of the donor, acceptor and complex.[9]

(9) The reaction in solution is not simply

$$\text{Donor} + \text{Acceptor} \rightleftarrows \text{Complex}$$

but

$$\text{Donor}\cdot S\cdot x + \text{Acceptor}\cdot S\cdot y \rightleftarrows \text{Complex}\cdot S\cdot z + (x + y - z)S$$

where S is the solvent and x, y and z are the numbers of solvent molecules solvating each species, respectively.

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[COMMUNICATION NO. 38 FROM THE DEPARTMENT OF BIOPHYSICS, FLORENCE R. SABIN LABORATORIES, UNIVERSITY OF COLORADO MEDICAL CENTER]

Kinetics of the Antigen–Antibody Reaction. Effect of Salt Concentration and *p*H on the Rate of Neutralization of Bacteriophage by Purified Fractions of Specific Antiserum[1a,b]

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The kinetics of neutralization of bacteriophage T2r$^+$ by purified fractions of specific antiserum indicate an electrostatically controlled diffusion process. The effects of salt concentration and *p*H on the rate of neutralization are interpreted in terms of changes in the electrostatic interactions between oppositely charged antigen and antibody combining sites, modified by changes in collision frequency between virus particles and antibody molecules. It appears that one or more carboxylate groups are involved in the antigen–antibody bond.

Introduction

Jerne[2] and Jerne and Skovsted[3] first reported that the rate of neutralization of bacteriophage by specific antiserum is considerably increased when the salt concentration is lowered. This has been confirmed by others.[4,5] The authors have made a preliminary report[6] of studies dealing with the effect of salt concentration and *p*H on the kinetics of neutralization of T2r$^+$ by purified fractions of specific antiserum, prepared by the method of electrophoresis–convection. These studies are reported in detail in the present communication.

Experimental

Materials.—Bacteriophage T2r$^+$ and *Escherichia coli* B were used in these experiments. Phage lysates were diluted in distilled water and incubated several hours at 37° in order to activate inhibited viruses.[7] Two different rabbit anti-T2 sera were studied: Serum I was a pooled sample from two animals, while Serum II was obtained from a single animal.

Fractionations.—The details of construction and operation of the electrophoresis-convection apparatus have been described previously.[8] Each of the antisera was carried through seven or nine successive stages of fractionation in the cold, the material from the bottom reservoir of the electrophoresis-convection cell at the end of each stage serving as the starting material for the succeeding stage. The successive steps of fractionation, which were carried out in buffers of ionic strength 0.1 and *p*H values shown in Table I, are designated as stage 1,2, etc.; and the fractions removed from the upper reservoir at the end of each stage are designated as top 1, 2, etc. The material taken from the bottom reservoir after the last stage was further separated into a globulin and albumin fraction (fractions BG and BA, respectively) by salting out with $(NH_4)_2SO_4$ at *p*H 7. A portion of each fraction was dried by lyophilization and used for electrophoretic analysis. The remainder was sterile-filtered and stored at 2° for subsequent immunological testing.

Electrophoretic analyses were carried out using the conventional moving boundary method.

Immunological Tests.—In the absence of the complication of serum activation of inhibited virus particles, neutralization of 90–99% of bacteriophage T2 by a large excess of specific antiserum follows the first-order kinetic[3,9] law $\mathrm{dln}\ V/dt = k(1/D)$ with a Q_{10} of 1.4. The symbol V represents the number of viable viruses remaining at time t after mixing with antiserum; D, the antiserum dilution in the reaction mixture; and k, a specific rate constant independent of the serum dilution. Whole antiserum and each of its fractions were tested for their ability to neutralize phage, with the aid of the plaque counting technique as described previously.[4] Routinely the virus–antibody reaction mixtures contained about 10^8 virus particles and 3–25 γ of serum protein per ml., and all tests were carried out under conditions of large excess of antibody. Dilutions of the serum fractions in media of low salt concentration were made immediately before testing for neutralizing activity.[10] Activated phage-lysates and purified viruses gave the same result. Heating the antiserum at 56° for 30 minutes had no effect on the rate at which it neutralized viruses; adsorption of the antiserum with either intact or ultrasonically lysed *E. coli* also had little or no effect upon the rate. Control tubes contained phage without antibody in solutions of the same salt concentration and *p*H as the reaction mixture.

(1) (a) This investigation was supported in part by a research grant from the National Institute of Arthritis and Metabolic Diseases of the National Institute of Health, Public Health Service; in part by an institutional grant from the Damon Runyon Fund and the American Cancer Society; and in part by research contract No. AT(11-1)-269 with the Division of Biology and Medicine, Atomic Energy Commission. (b) Some of this work was presented at the 126th Meeting of the American Chemical Society, New York, N. Y., September, 1954.

(2) N. K. Jerne, *Nature*, **169**, 117 (1952).

(3) N. K. Jerne and L. Skovsted, *Ann. Inst. Pasteur*, **84**, 73 (1953).

(4) J. R. Cann and E. W. Clark, *J. Immunol.*, **72**, 463 (1954).

(5) A. R. Fodor and M. H. Adams, *ibid.*, **74**, 228 (1955).

(6) J. R. Cann and E. W. Clark, *Biochem. Biophys. Acta*, **18**, 429 (1955).

(7) B. P. Sagik, *J. Bacteriol.*, **68**, 430 (1954).

(8) J. R. Cann and J. G. Kirkwood, *Cold. Spring Harbor Symposia Quant. Biol.*, **14**, 9 (1950); J. R. Cann, D. H. Campbell, R. A. Brown and J. G. Kirkwood, THIS JOURNAL, **73**, 4611 (1951).

(9) In contrast to the case[4] of T4, the Q_{10} for the neutralization of T2 is the same in 3×10^{-3} as in 0.15 *M* NaCl.

(10) Under certain conditions the neutralizing activity of unfractionated antiserum changes in a rather complicated manner during incubation at low salt concentrations.[4,6] While the serum fractions behave in a less complex fashion, prolonged incubation in media of low salt concentration does result in some loss of neutralizing activity.

TABLE I

ELECTROPHORETIC COMPOSITION AND IMMUNOLOGICAL ACTIVITY OF FRACTIONS OF RABBIT ANTI-PHAGE SERUM

Fraction	*p*H of fractionation	Electrophoretic composition[a] % of fraction Albumin	α-Globulin	β-Globulin[b]	γ-Globulin	Mobility[a] $-10^5 \times \mu$, cm.^{2} sec.$^{-1}$ volt^{-1} γ-globulin	Specific rate of virus neutralization at 37°, *p*H 7 and ionic strength 0.15[e] min.$^{-1}$ (mg. fraction per ml.)$^{-1}$
(A) Serum I							
Unfractionated Serum		65	6	16	13	1.54	2.4
Top 1	7.5				100	1.09	12
Top 2	7.0				100	1.39	13
Top 3	6.5			7	93	1.48	17
Top 4	6.0			24	76	1.81	15
Top 5	6.0	15	8	22	55	1.98	13
Top 6	5.4	44	6	33	17	2.08	4.0
Top 7	5.3	51	11	28	10	2.1 ± 0.1	1.1
BG-7		5	$\alpha_1 = 57$ $\alpha_2 = 27$	11[c]			1.8
BA-7		87	8	5			<0.0005
(B) Serum II							
Unfractionated serum		69	10	10	11	1.59	3.4
Top 1	8.2				100	0.91	15
Top 2	7.5	2		3	95	1.36	22
Top 3	7.0	3		3	94	1.43	23
Top 4	6.5	2		2	96	1.71	17
Top 5	6.0	9		19	72	1.93	8.6
Top 6	5.9	13		25	62	2.01	7.9
Top 7	5.9	24		29	47	2.32	4.2
Top 8	5.4	49		37[d]	14	2.2	3.4
Top 9	5.3	60		34[d]	6	2.1	0.077
BG-9		49	$\alpha_1 = 35$ $\alpha_2 = 12$	4[d]			0.40
BG-9 Reptd. with salt		22	$\alpha_1 = 49$ $\alpha_2 = 18$	11[d]			1.3
BA-9		94	4	2			<0.01

[a] Barbital buffer *p*H 8.6, ionic strength 0.1, protein concentration 1%. [b] Mobilities of β-globulins ranged from -3.4×10^{-5} to -3.8×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}. [c] Mobility of -2.7×10^{-5}. [d] Mobilities ranged from -2.7×10^{-5} to -3.1×10^{-5}. [e] Addition of 700 γ of normal serum protein to each ml. of the virus-serum fraction reaction mixtures had no effect upon the rates of neutralization.

Results

The electrophoretic compositions and immunological activities of the unfractionated sera and the various fractions are presented in Table I. The immunological activities were determined at *p*H 7 in buffered 0.15 *M* saline and are expressed in terms of specific rates of virus neutralization. All of the globulin fractions showed virus neutralizing activity, but correlation of electrophoretic composition and neutralizing activity indicates that the virus-neutralizing antibodies are associated principally with γ-globulins. The γ-globulins in the various fractions possess different mean electrophoretic mobilities, but as shown in Fig. 1A the specific neutralizing activities (as determined under these conditions of salt concentration and *p*H) of γ-globulins of different mobilities vary but slightly from one to the other. The possibility that antibody is also associated with some other electrophoretic component of the serum has not been excluded. Thus, for example, fraction BG-7 of Serum I which contained 5% albumin, 84% α-globulin, and 11% of a component with electrophoretic mobility intermediate between those of β- and γ-globulins but no detectable γ-globulin, neutralized virus. The rate of neutralization by this fraction was about 15% of that obtained with fractions containing only γ-globulin.

The rates of virus neutralization at *p*H 7 were also measured at a salt concentration of 3×10^{-3} *M* NaCl. The rate of neutralization by unfractionated serum was approximately 30 times faster in 3×10^{-3} *M* than in 0.15 *M* NaCl. Figures 1B and 1C show that whereas some of the serum fractions also showed a large increase in rate of neutralization on lowering the salt concentration, others showed little or no increase in rate, while with still others, the reaction was strongly inhibited at low salt concentration. There is a correlation between the mean isoelectric points of the fractions[11] and the effect of salt concentration on their rates of neutralization in neutral solution: the more acid the isoelectric *p*H, the less the rate is increased on

(11) The theory of transport of an electrophoretically heterogeneous protein in an electrophoresis–convection channel[12] predicts that transport in the apparatus will proceed to a stationary state in which the top fraction is isoelectric at the operating *p*H. Departures from the isoelectric condition are to be expected if some of the components of the heterogeneous protein produce different density increments per unit weight or possess different diffusion constants. The theoretical isoelectric condition of the top fraction at the operating *p*H was realized at least in the case of Top 1 of Serum I. This fraction was obtained by operating at *p*H 7.5 and was found to be isoelectric at *p*H 7.56. For this determination electrophoretic mobilities were determined in 0.3% protein solutions in cacodylate–cacodylic acid and barbiturate–barbituric acid buffers of ionic strength 0.01.

(12) J. G. Kirkwood, J. R. Cann and R. A. Brown, *Biochem. Biophys. Acta*, **5**, 301 (1950); **6**, 606 (1951).

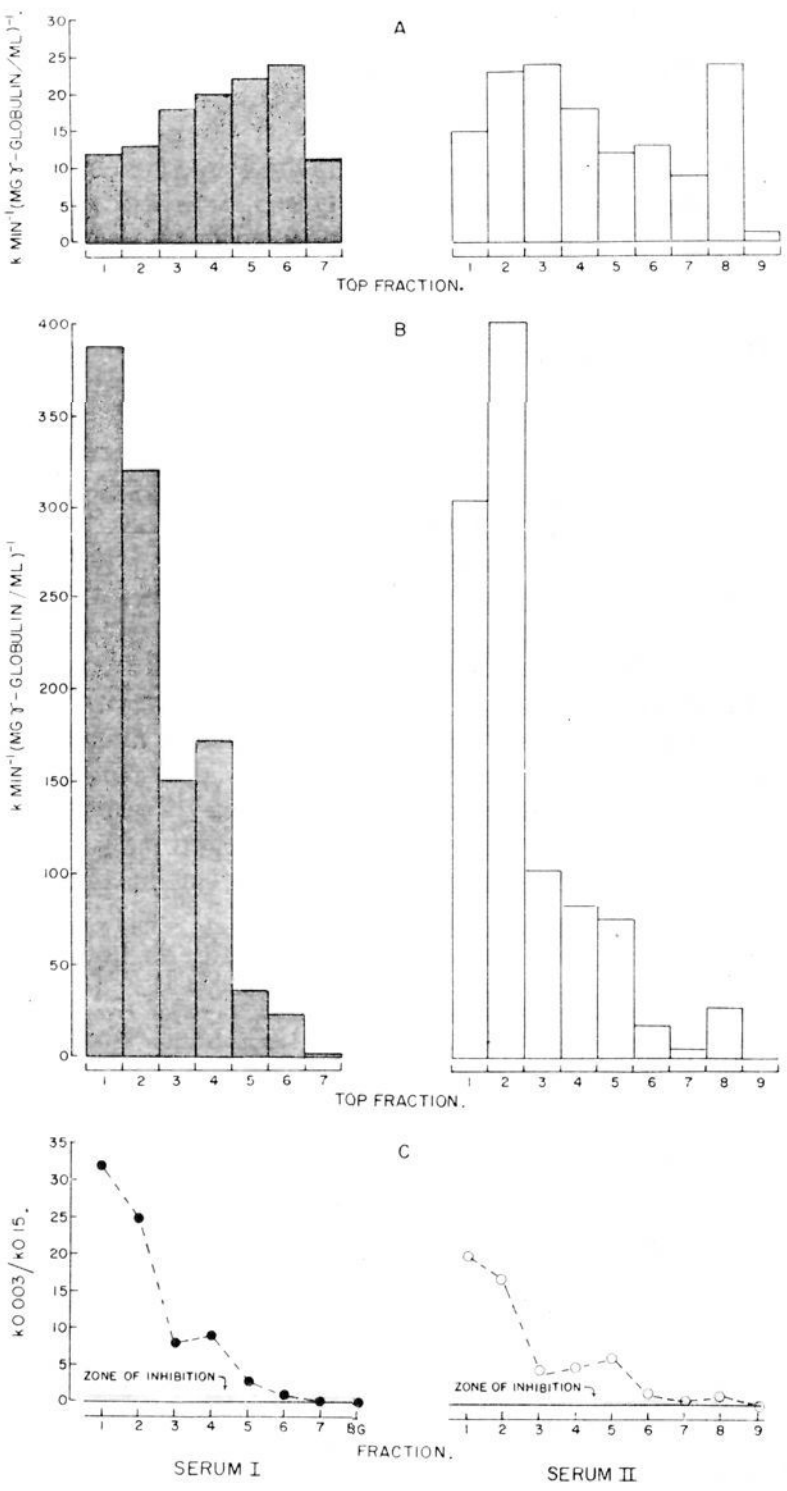

Fig. 1.—Effect of salt concentration on specific rate of neutralization of phage by serum fractions at *p*H 7: A, 0.15 *M* NaCl; B, 3×10^{-3} *M* NaCl; C, ratio of rate in 3×10^{-3} *M* NaCl to rate in 0.15 *M* NaCl, fraction 6 and beyond fall close to or within the zone of inhibition; rates of neutralization were determined at 37°.

lowering the salt concentration, neutralization by the most acidic fractions being inhibited at low salt concentrations. The following observations indicate that the different behaviors cannot be attributed to some action of the different non-specific proteins in the fractions: (1) The rates of neutralization at low salt concentration by mixtures of Top 1 with Top 5 and Top 7, Serum I, were additive and (2) the addition of as much as 1000γ of normal serum proteins to each ml. of the various reaction mixtures had relatively little or no effect on the dependence of the rates of neutralization upon salt concentration.

Whereas the rate of virus neutralization in 0.15 *M* NaCl is insensitive to the hydrogen ion concentration over a rather wide range of *p*H, the rate is extremely sensitive to hydrogen ion concentration in 3×10^{-3} *M* NaCl. For example, at the lower salt concentration Top 1, Serum I, showed about a 4-fold increase in rate on going from *p*H 7 to 5.7; Top 5, a 20-fold increase; and BG-7, a 120-fold increase. Detailed studies of the effect of *p*H on the rate of neutralization were carried out on fractions Top 1 and 5. The results of these experiments, which are presented in Fig. 2, show that at low salt concentration the rate of neutralization increases many fold on going from *p*H 7–8 to about 5.5, but that further lowering of the *p*H results in strong inhibition of the neutralization reaction. The *p*H at which inhibition begins is insensitive to the isoelectric point of the fraction. These results also show that the rate of neutralization is sensitive to salt concentration even at the isoelectric *p*H of the serum fractions.

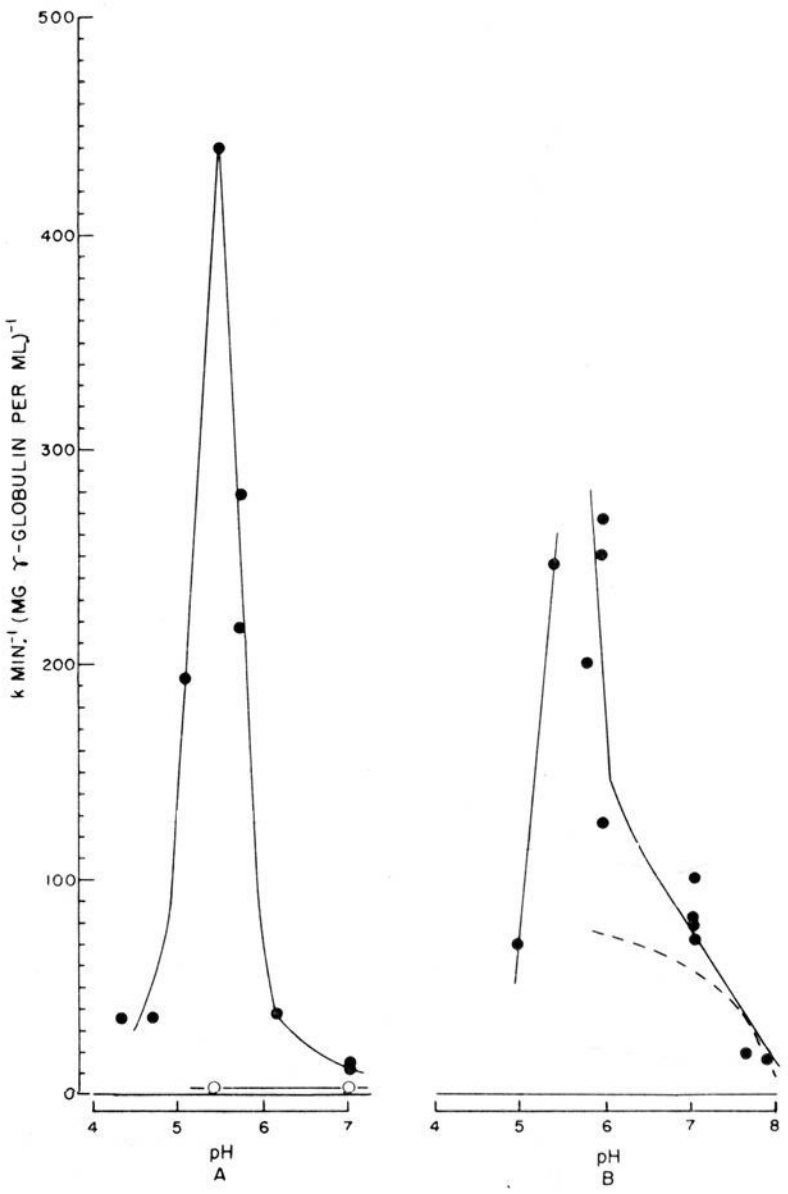

Fig. 2.—Effect of *p*H on specific rate of virus-neutralization in 3×10^{-3} *M* NaCl: A, Top 5, Serum I; B, Top 1, Serum I, isoelectric *p*H 7.56. Rates of neutralization were determined at 0°. Broken curve in A was calculated using the methods of Verwey and Overbeek (see text).

The quantitative relationships between rate of virus neutralization by Top 1, Serum I, and salt concentration, over the range of ionic strengths 0.15 to 3×10^{-3}, have been determined at *p*H values acid to the isoelectric point of the fraction using several different electrolytes. These relationships are shown in Fig. 3 where $\log k - \log k_{\kappa=0}$ is plotted against the square root of the ionic strength, $\Gamma/2$. The symbol k is the specific rate of neutralization, and $k_{\kappa=0}$ is the rate at zero ionic strength. Values of $k_{\kappa=0}$ are given in the

legend to Fig. 3. Binding of H^+, Mg^{++} and ClO_4^- to one or both of the reactants is reflected in increased slopes of these plots.

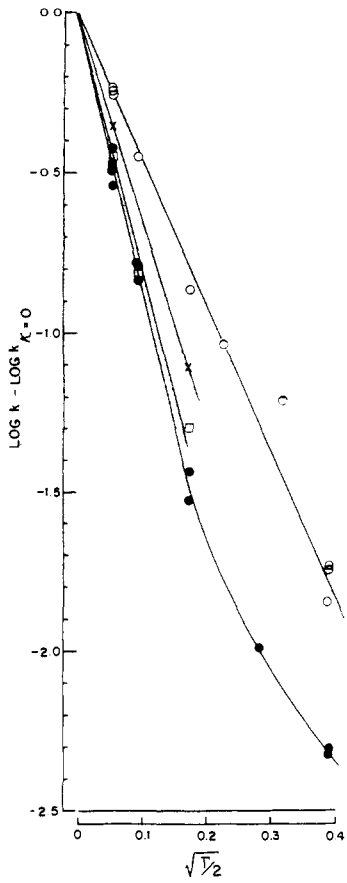

Fig. 3.—Effect of ionic strength, $\Gamma/2$, on rate of virus-neutralization by Top 1, Serum I: O, NaCl buffered at pH 7, $k_{K=0}$ = 700; x, $NaClO_4$ pH 7, $k_{K=0}$ = 700; $MgCl_2$ pH 7, $k_{K=0}$ = 700; NaCl pH 5.7, $k_{K=0}$ = 3800. Rates of neutralization were determined at 37°.

Control experiments with bacteriophage T5 have shown that anti-T2 bodies do not lose their specificity under conditions of pH and salt concentration which yield the maximal rate of T2 neutralization. It has also been shown that normal serum proteins do not neutralize T2 under these conditions. The rate of virus neutralization at pH 7 and ionic strength 0.15 is not affected by the addition of the sequestering agent ethylenediaminetetraacetic acid to the virus–antibody reaction mixture, which would seem to eliminate possible involvement of heavy metals in the neutralization reaction.

Discussion

The kinetics of neutralization of phage by specific antibody indicate that the rate-limiting step of virus-neutralization is the reaction of one antibody molecule with one virus particle. Further, it appears that this reaction is an electrostatically controlled diffusion process. The effect of salt concentration on the rate of neutralization is in qualitative agreement with the predictions of the Brönsted theory of the primary salt effect on reaction kinetics in solution.[13] This theory predicts, for example, that the rate of reaction between oppositely charged ions will be increased as a result of lowering the ionic strength of the reaction mixture. The increased rate results from increased electrostatic attraction between the ions, which in turn results from decreased shielding of the ionic charges by the diffuse double layer. Such changes in rate were observed in the phage-antibody reaction at pH values between the isoelectric points of the phage[14] and serum fractions, where the phage and antibody are oppositely charged (see Fig. 3 for example). The method of Verwey and Overbeek[15,16] should permit one to estimate the changes in rate of reaction resulting from changes in collision frequency between charged particles with large surface potentials. However, an approximate computation using this method (net charge densities and zeta potentials derived from electrophoretic measurements[17]) failed to account, by an order of magnitude, for the greatly increased rates of neutralization of phage observed at low ionic strengths and the pH values under consideration. It would thus seem that it is not the electrostatic interaction between the net charges on the two particles which controls the rate of reaction at these pH's. Interpretation of these results is not entirely clear and may be complicated by factors such as interaction of the fluctuating multipoles of macromolecules recently described by Kirkwood[19] and his co-workers. It is suggested, however, that the rate of the virus–antibody reaction under the given conditions of pH is largely controlled by electrostatic interaction between oppositely charged, specific antibody and antigen combining sites rather than by interaction between the net

(13) E. S. Amis, "Kinetics of Chemical Change in Solution," The Macmillan Co., New York, N. Y., 1949, Chapter IV.

(14) The isoelectric point of phage is about pH 4.

(15) E. J. W. Verwey and J. Th. G. Overbeek, "Theory of the Stability of Lyophobic Colloids," Elsevier Publishing Co., New York, N. Y., 1948.

(16) The number of collisions per second, ν, between two charged particles of radii a_1 and a_2 in solutions of concentration n_1 and n_2, is given by the usual Smoluchowski equation modified to take into account electrostatic interaction between the colliding particles[15]

$$\nu = \frac{(1/a_1 + 1/a_2)}{\int_{a_1+a_2}^{\infty} e^{U/kT}\frac{dx}{x^2}} \times \frac{2kT}{3\eta} n_1 n_2$$

The symbol U represents the potential of interaction; and x, the distance between the centers of the colliding particles. The other symbols have their usual meanings. If, for example, the two particles carry net charges of the same sign, then U will be a repulsive potential and the collision frequency will be smaller than expected from simple diffusion theory. An approximate expression for U was obtained by assuming that the electrical potential of the double layer, ψ, between the two particles can be taken as the sum of the electrical potentials due to the two particles separately

$$U = \sigma_1 \int^{A_1} \psi_2 dA + \sigma_2 \int^{A_2} \psi_1 dA$$

The symbol σ represents the surface charge density; and A, the area of the particle. Integration was carried out assuming that the interaction is between large particles with arbitrary surface potential (taken as the zeta potential) and thin double layers.

The charge densities and zeta potentials of the particles were estimated from electrophoretic data.[17]

In making the calculations shown in Fig. 2B the rate at a given pH was obtained from the rate observed at the isoelectric pH by suitable correction for electrostatic interaction between net charges on the reacting particles. This assumes that the fraction of collisions leading to fruitful union between virus and antibody is independent of pH.

The peculiar morphology of the phage makes these computations awkward; however, essentially the same results were obtained when one considered collisions between antibody molecules and viruses represented as spheres of either 270 or 100 Å. radius, one-half the thickness of the phage tail.

(17) The charge on the antibody molecules was estimated from electrophoretic mobility measurements on Fraction Top 1, Serum I, in uni-univalent buffers of ionic strength 0.01. In the case of the virus, the reasonable assumption was made that the mobilities of bacteriophage T2 are about the same as those[18] of T6 under the same conditions of pH and ionic strength. The charge density was assumed to be uniform over the surface of the phage.

(18) F. W. Putnam, L. M. Kozloff and J. C. Neil, *J. Biol. Chem.*, **179**, 303 (1949).

(19) J. G. Kirkwood and J. B. Shumaker, *Proc. Natl. Acad. Sci.*, **38**, 855, 863 (1952); S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, *ibid.*, **41**, 710 (1955).

charges carried by the two particles. Such interaction between local areas on the particles (in addition to steric factors) might, for example, determine the fraction of total collisions leading to fruitful union. This interpretation is consistent with the observation that the rate of virus-neutralization is sensitive to salt concentration even at the mean isoelectric *p*H of the serum fractions.

Whereas electrostatic interactions between the net charges carried by viruses and antibody molecules appear to be only of secondary importance at *p*H values between the isoelectric points of the two particles, such interactions appear to be of considerable importance in controlling the rate of reaction at low salt concentration and *p*H values alkaline to the isoelectric point of both the phage and the serum fractions. At these *p*H values both the viruses and antibody molecules are negatively charged and the resultant coulombic repulsions decrease the collision frequency between virus and antibody. Approximate computations of the changes in the rate of reaction as a result of changes in collision frequency have been made using the method of Verwey and Overbeek.[15,16] These computations, which are shown in Fig. 2B, indicate that the decrease in rate of reaction with increasing *p*H alkaline to the isoelectric point of the antibody results from decreased collision frequency. This then affords an explanation for the correlation between the mean isoelectric point of the serum fractions and the effect of salt concentration on their rate of neutralization in neutral solutions. It appears that if the net charges are of the same sign and become sufficiently large, then the rate of union of virus and antibody is inhibited rather than increased at low salt concentration due to electrostatic repulsion between the particles.

The sensitivity of the rate of virus-neutralization at low salt concentration to hydrogen ion concentration can also be explained in terms of electrostatic forces. Thus, the increase in rate on going from *p*H 7 to 5.5 appears to be due to increased coulombic attraction between combining sites as a result of binding of protons by some group or groups perhaps imidazoles which bind protons in this *p*H range, in one or both of the sites or their immediate vicinity. That binding of protons by these groups is not, in itself, essential for the reaction is indicated by the fact[20] that the rate of neutralization at high salt concentration is insensitive[21] to *p*H over the range 5 to 10. Inhibition of the neutralization reactions at *p*H below 5.5 is attributed to the binding of protons by one or more carboxylate groups in one or both of the sites. That these carboxylate groups are involved in the antigen–antibody bond is indicated by the fact[20] that neutralization is also inhibited on going from *p*H 5 to 4 at high salt concentration.

Our conclusions regarding the nature of antigen and antibody combining sites are not inconsistent with those of other workers. Thus Kabat[22] concludes that the antibody combining site on human antidextrans had dimensions complementary to a chain of at least three α-D-glucosidopyranose units and probably to part or all of a fourth unit. Steiner[23] has found that the effect of salt concentration on the molecular weight of soluble antigen–antibody complexes in the system, human plasma albumin and its rabbit antibodies, is consistent with the idea that the two combining sites are oppositely charged. And finally, Singer and Campbell[24] and Singer, Eggman and Campbell[24] present strong evidence that a single carboxylate group is involved in every antigen—antibody bond in the systems bovine serum albumin and ovalbumin and their respective rabbit antibodies.

Acknowledgment.—We wish to thank Professor John G. Kirkwood for reviewing our manuscript.

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(20) G. M. Kalmanson, A. D. Hershey and J. Bronfenbrenner, *J. Immunol.*, **45**, 1 (1942).

(21) The distribution of rates of neutralization among the serum fractions at *p*H 7 and in buffered 0.15 *M* NaCl, Fig. 1A, probably approximates rather well the distribution of antibody among the fractions.

(22) E. A. Kabat, THIS JOURNAL, **76**, 3709 (1954).

(23) R. F. Steiner, *Arch. Biochem. Biophys.*, **55**, 235 (1955).

(24) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 3504 (1955); S. J. Singer, L. Eggman and D. H. Campbell, *ibid.*, **77**, 4855 (1955).