NEUTRALIZATION OF VIRUSES BY HOMOLOGOUS IMMUNE SERUM

II. Theoretical Study of the Equilibrium State

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Quantitative studies on the neutralization of animal viruses by homologous antibody usually have been carried out by measuring the minimum amount of specific immune serum required to neutralize varying quantities of the agent. In the preceding paper (1), results obtained in numerous experiments of this type are reviewed and some additional data are presented. In experiments on the neutralization of bacterial virus T_3 by a method similar to that used for animal viruses, it was found that the results could be predicted by an empirical equation (1). The equation related the proportion of virus which remained unneutralized to the concentration of antibody added and involved two arbitrary constants.

The object of this paper is to present a simple theoretical model from which an equation for the equilibrium state of the virus neutralization reaction may be deduced. The equation is identical in form with that fitted to the results of experiments with bacterial virus T_3 (1), and can be applied to available data on the neutralization reaction with animal viruses.

It is postulated that, in a mixture containing many virus particles and homologous antibody, there exist several combinations represented by:

$$v,\ va\cdots va_x\cdots va_n\cdots$$

which indicate virus particles v onto which there are adsorbed various numbers of units of antibody a ranging from 1 to n. Combinations containing one unit of antibody and more than one virus particle are assumed to be rare. The frequency of combinations in each category will be related to the number of antibody units on each virus particle. No techniques are available to measure the frequencies of such categories individually, nor can the distribution be predicted certainly on theoretical grounds. The possible results, if the distribution were of binomial or Poisson form, were investigated and were found not to agree with the experimental data.

Fig. 1 represents a hypothetical distribution in which F is the number of virus particles in each category and A is the number of antibody units carried

per particle. If few virus particles carry more than n antibody units, the sum of the values of F between $A = o$ and $A = n$ will be approximately equal to the whole distribution; or, if the process is considered as successive additions of antibody units to virus, the addition process may be thought of as almost stopping when n units of antibody have been added. If $(n - x)$ is the largest number of antibody units a virus particle can carry and remain infective, then the shaded area, *i.e.* the sum of the values of F between $A = o$ and $A = (n - x)$, will represent the number of infective particles in the mixture.

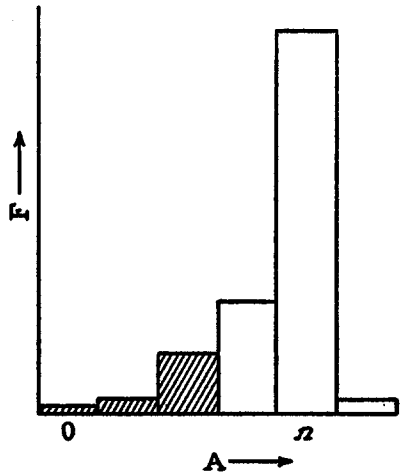

FIG. 1. Hypothetical frequency distribution of virus particles carrying various numbers of antibody units. F = number of virus particles in each category. A = number of antibody units carried per virus particle.

It is not easy to make a simple analytical treatment of such a distribution, but a model suggested by previous theoretical studies carried out by Heidelberger and Kendall (2, 3) and Jerne (4) on other antigen-antibody reactions has proven useful. It is assumed that the reaction by which antibody is added to virus obeys the law of mass action. It may readily be shown (*cf.* Appendix) that

$$[va_{(n-x)}]\,[a]^x = K[va_n]$$

in which square brackets indicate the concentration of the virus-antibody combinations of the type enclosed within them.

Because the concentration of neutralized virus particles was greatly in excess of the concentration of infective particles in the range studied, it is assumed that $[va_{(n-x)}]$ is considerably greater than $[va_{(n-x-1)}]$ so that, within the limits of accuracy of the experimental procedure, one may regard the concentration of the first infective combinations of virus and antibody as negligible compared

with the concentration of the last combination in the series, *i.e.*, $va_{(n-x)}$. This is equivalent to regarding the concentrations of successive combinations of virus and antibody as terms of a rapidly expanding series. Similarly, it is assumed that $[va_n]$ is nearly equal to the concentration of all the virus particles in the system. In order to produce this state, a large excess of antibody is needed so that the final concentration of free antibody is nearly equal to the total concentration of antibody at the beginning of the reaction.

Let $\mathbf{v}$ = concentration of infective virus in mixture $\simeq [va_{(n-x)}]$; $\mathbf{V}$ = concentration of virus particles added to the mixture $\simeq [va_n]$; $\mathbf{A}$ = concentration of antibody added to the mixture $\simeq [a]$; then, substituting in (1)

$$\mathbf{v} \cdot \mathbf{A}^x = \mathbf{K} \cdot \mathbf{V} \tag{2}$$

or

$$\log \mathbf{V} = \mathbf{x} \cdot \log \mathbf{A} + \log \mathbf{v} - \log \mathbf{K} \tag{3}$$

It is probable that neither virus nor antibody is entirely homogeneous. Therefore, some virus particles will be neutralized when carrying $(n - x)$ units of antibody and others when carrying $(n - x + 1)$ units. As a result, values of x may be found experimentally which are intermediate between two integers.

Comparison of Theoretical Results with Experimental Data.—Equation (3) has the same form as the equation fitted empirically to the data on the neutralization of bacterial virus T_3 given in the preceding paper (1). When $\mathbf{x}$ is taken as equal to 2 and a suitable value is used for log $\mathbf{K}$, the equation fits almost all the experimental data found with T_3 as well as the neutralization lines derived therefrom (1). At extreme values, the experimental data appeared to deviate from a straight neutralization line. This was observed also in unreported experiments with the NWS strain of influenza A virus and by others (5) with herpes simplex virus. Such a deviation is to be expected because equation (3) will hold only while the simplifying assumptions used in deriving it are applicable. For instance, when small amounts of virus are neutralized by but little antibody, the combinations $va_{(n-x+1)} \ldots va_{(n-1)}$ will not be negligible compared with va_n and less antibody will be required than is predicted by the equation.

This theoretical treatment has been applied also to the data on the neutralization of animal viruses given in the accompanying paper (1). The value of $\mathbf{v}$ was taken to be the number of infective doses for the chick embryo (EID_{50}) contained in one infective unit of virus as determined in the host-cell system used. For example, there are approximately 7,000 EID_{50} of the NWS strain of influenza A virus in one infective unit (MS_{50}) as determined in the mouse lung (1). Therefore, the mixture of serum and virus which yields a mean score of 50, *i.e.* 1 MS_{50}, in the mouse lung contains 7,000 EID_{50} of infective virus. Thus, although neutralization experiments were carried out in the mouse lung, the

quantity of virus added and the quantity which remained infective can be expressed in chick embryo infective doses. For **x**, the slope of the neutralization

TABLE I

Application of Theory to the Results of Neutralization Experiments with Influenza and Newcastle Disease Viruses

Virus	Immune serum	Procedure	Criterion for 50 per cent infectivity end point	Experimental values*				Computed value‡
				Log V	Log A	x	Log v	log K
NWS§	R1	Neutralization on chorio-allantoic membrane	Death‖	4.5	2.4	2.6	0	1.7
"	"	Neutralization in allantoic cavity	HA¶	4.5	3.0	4.7	1.0	10.6
"	"	Neutralization in mouse brain	Death	5.5	2.4	1.0	4.0	0.9
"	"	Neutralization in mouse lung	Lung lesions	6.1	2.0	1.2	4.5	0.8
"	"	Hemagglutination-inhibition	—	6.5	2.5	1.3	5.9	2.6
NDV	R6	Neutralization in allantoic cavity	HA	4.5	2.9	5.8	0	12.3
"	"	" "	Death	4.5	3.1	3.6	0	6.7
"	R3	" "	HA	3.5	2.8	4.1	0	8.0
"	"	Neutralization on chorio-allantoic membrane	Death	3.5	3.3	1.6	0	1.8
WS§	R2	Neutralization in allantoic cavity	HA	3.7	2.5	3.6	0	5.3
"	"	Hemagglutination-inhibition	—	4.3	1.8	(1)	3.7	1.2

* **V**, **A**, and **x** were determined from regression equations fitted to the experimental points. The values of **x** are given in Tables I and III in the accompanying paper (1). **V** was measured in terms of EID_{50} units in all instances. **A** was measured in arbitrary units of immune serum taking one volume of a 10^{-5} dilution as unity. **v** was computed as indicated in the text.

‡ Computed by means of equation (3).

§ Influenza A virus strains.

‖ Death of embryos 2 to 6 days after inoculation.

¶ HA, hemagglutination after 2 days' incubation.

line (1), determined by experiment, was employed. Values of **V** and **A** can be found from the appropriate regression equation (1).

In order to test the applicability of equation (3), the value of log **K** was calculated from the data obtained in experiments with animal viruses. The results obtained in various neutralization experiments (1), in which comparable sera and techniques were used, are given in Table I. High values for **K** were

found for experiments in the allantoic cavity and low values for experiments on the chorioallantoic membrane as well as in the other host-cell systems. As shown in Fig. 2, in which log **K** is plotted against **x**, the results for the allantoic cavity experiments appear to fall near a straight line, but this is probably not of great importance for the following reason: When **x** is large, the value of log **K** is largely fixed by **x** and the apparent linearity is that which would be ex-

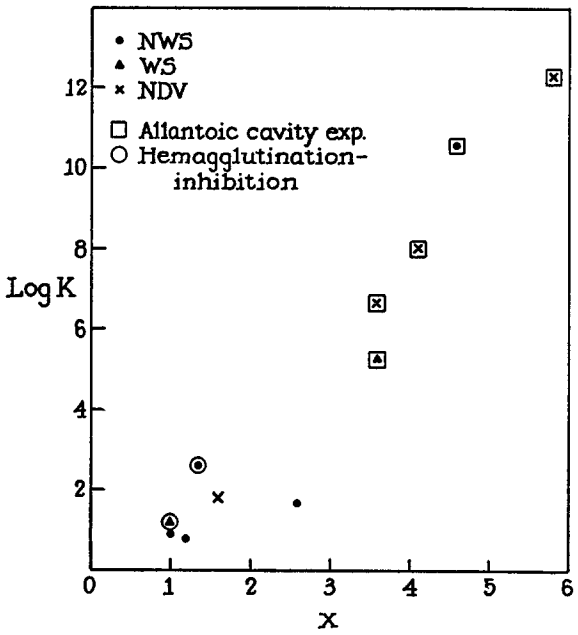

FIG. 2. Values of log **K** computed by means of equation (3) from the results of neutralization experiments with influenza (NWS and WS) and Newcastle disease (NDV) viruses (1). **x** = slope of neutralization line determined by experiment.

pected if the average amount of neutralization were constant and various errors in the estimation of **x** had occurred. There seems to be little upward or downward trend in the smaller values of log **K** as **x** varies. This is consistent with $k_{(n-1)}$ being fairly large and $k_{(n-2)}$ being smaller, *i.e.*, the antibody added in the last steps of the reaction series is added less readily than in earlier steps. On this basis, in order to explain the much higher values for log **K** obtained in allantoic cavity experiments, it would be necessary to assume that other k values, *i.e.* $k_{(n-4)}$ and $k_{(n-5)}$, were larger than $k_{(n)}$ and $k_{(n-1)}$. This implies that the first additions of antibody to virus occur *less* readily than later additions which seems improbable.

An alternative explanation is that, in the allantoic cavity, virus is neutralized mainly by a second kind of antibody present in lower concentration or with a

different equilibrium reaction or both. In other words, values for log **K** fall into two groups because a different neutralization reaction occurs in the allantoic cavity than on the chorioallantoic membrane or in the other host-cell systems studied (1). In support of this hypothesis, there is experimental evidence (6) indicating that the antibody which causes neutralization of virus in the allantoic cavity is not identical with that which causes hemagglutination-inhibition *in vitro*. Also it should be pointed out that, as shown in Table I, log **K** values calculated from *in vitro* hemagglutination-inhibition experiments are close to those found in neutralization experiments with the same sera carried out in host-cell systems other than the allantoic cavity.

DISCUSSION

Andrewes and Elford (7) found that, when varying amounts of bacteriophage were added to a given dilution of immune serum, the proportion of the virus added which remained infective was constant irrespective of the amount added. This relation they termed the "percentage law." Burnet *et al.* (8) showed that this finding was in agreement with the theory that antibody was adsorbed to the virus surface by a reversible process obeying simple quantitative laws. The theory of Burnet *et al.* (8) gave results similar to those of a special case, *i.e.* in which **x** is equal to one, of the theory developed in this communication. As a result of this limitation, however, the earlier theory does not account satisfactorily for all the available data on the number of infective particles found when a constant amount of virus is allowed to react with varying concentrations of immune serum.

Kalmanson *et al.* (9) found that, with relatively dilute immune sera, the rate of neutralization of bacteriophage behaved like a first order reaction until neutralization was nearly complete. In fact, the neutralizing power of an anti-bacteriophage serum is commonly measured by means of the constant K of an exponential equation fitted to data from kinetic neutralization experiments (10, 11). This is not incompatible with the present theory if one assumes that the rate constants of the successive reactions increase progressively so that the $(n - x)$th reaction is approaching equilibrium, and therefore most of the virus added is combined with $(n - x)$ units of antibody, before combination between $va_{(n-x)}$ and antibody occurs.

The hypothesis that the equilibrium state of a virus neutralization reaction follows mass action laws is supported by the fact that such an equilibrium is, in fact, finally established (10, 12) and that to some extent it is reversible (1, 13). In developing a theory for specific antigen-antibody precipitation, Heidelberger and Kendall (2, 3) postulated that the reaction between protein or polysaccharide antigen and the corresponding antibodies obeyed mass action laws. They considered that both antigen and antibody were polyvalent with respect to each other and that an insoluble complex was formed after several

stages of competing bimolecular reactions. In quantitative investigations on the precipitin reaction, it is feasible to study mixtures of antigen and antibody in which an excess of either component is present (2, 3). In the virus neutralization reaction, this has not been possible. When mixtures of virus and immune serum, comparable to those used in neutralization experiments, are filtered or centrifuged to remove virus-antibody complexes, the amount of uncombined antibody recovered is closely similar to the total amount of antibody added (6, 7). Therefore, in the neutralization reaction with viruses, as usually carried out (1), there appears to be a great excess of antibody and only combinations of single virus particles with increasing amounts of antibody need be considered. The results of this study are to some extent parallel to those obtained by Jerne (4) who studied the neutralization of diphtheria toxin by antitoxin. He obtained the best fit to the experimental data by assuming that the concentration of free toxin was fixed by two successive equilibrium reactions in which antibody combined with toxin to form compounds containing either one or two antibody molecules for each toxin molecule.

No definite answer can be given to the question of what stops the series of additions of antibody to virus. It may be that the antibody receptors of the virus particle are eventually saturated. However, bacteriophage particles can take up many more antibody molecules (11, 12) than are required for neutralization, but the equilibrium constants of these later additions of antibody might be so much higher than those of the earlier additions that the late stages of the reaction could be neglected. Finally, it is known that aggregates form rapidly in antigen-antibody mixtures outside the range of visible precipitation (14, 15). If such aggregates form in mixtures of virus and immune serum, they may effectively stop any further addition of antibody molecules and so bring the reaction to a halt even though all receptor sites on the virus are still not covered with antibody.

SUMMARY

On the assumption that antibody is added to virus particles in a stepwise manner and that the reaction follows the law of mass action, a theoretical equation for the equilibrium state of virus neutralization reactions has been deduced.

The theory has been found to be in reasonable accord with the available data for the neutralization of bacterial viruses and can be applied also to data for the neutralization of animal viruses.

APPENDIX

If the first stage in the addition of antibody be represented as

$$v + a \rightleftharpoons va,$$

then, by mass action laws,

$$[v][a] = k_0[va]; \tag{i}$$

for later additions,

$$[va][a] = k_1[va_2] \tag{ii}$$

and

$$[va_2][a] = k_2[va_3]. \tag{iii}$$

Substituting from (i) in (ii), we have

$$[v][a]^2 = k_0k_1[va_2],$$

and similarly, in (ii) and (iii),

$$[va][a]^2 = k_1k_2[va_3].$$

For the general case we have

$$[va_{(n-x)}][a] = k_{(n-x)}\,[va_{(n-x+1)}],$$

and similarly, for successive additions up to

$$[va_{(n-1)}][a] = k_{(n-1)}\,[va_n].$$

Substituting as before between successive equations,

$$[va_{(n-x)}][a]^x = K[va_n],$$

in which $K = k_{(n-x)} \cdot k_{(n-x+1)} \cdot \ldots k_{(n-1)}$

BIBLIOGRAPHY

1. Tyrrell, D. A. J., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1953, **97,** 845.
2. Heidelberger, M., and Kendall, F., *J. Exp. Med.*, 1935, **61,** 563.
3. Heidelberger, M., and Kendall, F., *J. Exp. Med.*, 1935, **62,** 697.
4. Jerne, N. K., *Acta Path. Microbiol. Scand.*, 1951, suppl. No. 87.
5. Jawetz, E., and Coleman, V. R., *J. Immunol.*, 1952, **68,** 645.
6. Walker, D. L., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1950, **91,** 65.
7. Andrewes, C. H., and Elford, W. J., *Brit. J. Exp. Path.*, 1933, **14,** 367.
8. Burnet, F. M., Keogh, E. V., and Lush, D., *Australian J. Exp. Biol. and Med.*, 1937, **15,** 227.
9. Kalmanson, G. M., Hershey, A. D., and Bronfenbrenner, J., *J. Immunol.*, 1942, **45,** 1.
10. Hershey, A. D., Kalmanson, G. M., and Bronfenbrenner, J., *J. Immunol.*, 1943, **46,** 267.
11. Delbrück, M., *J. Bact.*, 1945, **50,** 137.
12. Hershey, A. D., Kalmanson, G. M., and Bronfenbrenner, J., *J. Immunol.*, 1943, **46,** 281.
13. Taylor, R. M., *J. Immunol.*, 1941, **40,** 373.
14. Gitlin, D., and Edelhoch, H., *J. Immunol.*, 1951, **66,** 67.
15. Goldberg, R. J., and Campbell, D. H., *J. Immunol.*, 1951, **66,** 79.