A QUANTITATIVE THEORY OF INFLUENZA VIRUS
HEMAGGLUTINATION-INHIBITION

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Viruses of the mumps-Newcastle-influenza group occupy a prominent position in current research on viruses because of their enzymatic activity against certain mucoproteins, which occur in nature as surface constituents of cells (e.g., red blood cells) and as dissolved components of biological fluids (so-called inhibitors of hemagglutination). Some aspects of the history of this development have been discussed in recent reviews (Svedmyr, 1949a; Briody, 1950; Burnet, 1951).

In previous studies (Lanni et al., 1951a,b; Lanni and Lanni, 1951) we have been concerned with complex changes in the shape of the hemagglutination-inhibition curve which occur when egg white inhibitor is treated with active influenza viruses. As further experiments progressed, it became evident that no satisfactory interpretation could reasonably be expected in the absence of an adequate analysis of the basic phenomenon of hemagglutination-inhibition by unmodified inhibitor. Since the key to this analysis was not discovered until after the period of experimentation had terminated, the desirability of certain experiments, e.g., the direct measurement of the rate of adsorption of virus onto red blood cells, failed to be anticipated.

In this paper there is developed and tested a quantitative theory of hemagglutination-inhibition phenomena observed with egg white inhibitor and heated (enzymatically inactive) swine influenza virus. The parameters of the theory are derived from experiments which constituted controls in the studies of the interaction of inhibitor with active (unheated) virus.

MATERIALS AND METHODS

Virus. Embryonated 11-day eggs were inoculated in the allantoic sac with egg-adapted swine influenza virus (Shope's strain 15), and the allantoic fluids were harvested after 40 hr at 35 C. Suitable fluids were pooled and stored at 4 C without preservative. In this form the virus possesses considerable inhibitor destroying activity and is referred to as active virus. For the present experiments, the virus was first mixed with 1 volume of 0.06 M phosphate buffer of pH 7.2 to 7.3 and 1 volume of 5.7 per cent sodium citrate solution and heated for 30 min at 53 C. Virus treated in this way is inhibited to high titer by egg white inhibitor and possesses no demonstrable inhibitor destroying activity. One hemagglutinating dose of virus is the amount needed to give a conventional end point of partial hemagglutination under standard conditions.

1 The experimental part of this investigation was carried out in the Virus Section, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, under a fellowship granted to the senior author.
Inhibitor. Egg white was obtained from fresh hens' eggs. Semipurified inhibitor was prepared from undiluted thick white by a single precipitation with 7 volumes of 0.1 M KH₂PO₄ and repeated extractions of the washed precipitate with 1 volume of 0.06 M phosphate buffer of pH 7.2 to 7.3 (Lanni et al., 1949). The experiments were carried out with two preparations, M37-PEII and M60-PEI, with standard inhibition titers 29,600 and 16,500, nitrogen contents 114 and 82 µg per ml, and standard purification factors 65 and 23, respectively. Solutions were preserved with 0.02 per cent merthiolate (Lilly) and stored at 4 C.

Red blood cells. A volume of 20 ml of chicken blood was collected by cardiac puncture in a syringe containing 5 ml of 2.5 per cent sodium citrate. The volume was brought to about 100 ml with chilled buffered saline, and the material was centrifuged 7 min at 1,600 rpm (600 X g). The cells were washed twice more with chilled saline. Then they were transferred to two 15 ml graduated tubes, diluted to 15 ml with chilled saline, and centrifuged 10 min at 1,200 rpm (320 X g). The packed cells were stored at 4 C, and samples were taken as needed over periods not exceeding 3 days. Routine 2.0 per cent suspensions (packed cells diluted with 49 volumes of buffered saline) contained on the average 1.12 X 10^9 red blood cells per ml.

Diluent. Buffered saline (0.81 per cent sodium chloride and 0.005 M phosphate at pH 7.2 to 7.3) was the diluent for all materials employed in the hemagglutination and hemagglutination-inhibition tests.

Glassware. A stock of reaction tubes, with O.D. 12.25 to 12.35 mm and wall thickness 1.00 ± 0.05 mm, was selected from a supply of Kimball Neutraglas tubes. Representative samples were found satisfactory in photometric tests with distilled water, potassium dichromate solution, and hemagglutinating mixtures set up at various ratios of virus to red blood cells.

Kimball Blue-Line Exax serological pipettes were used almost exclusively.

All glassware was cleaned with sulfuric acid-dichromate mixture, with thorough rinsing in tap and distilled water.

Photometer. The apparatus employed for the hemagglutination readings differed from those described by Hirst and Pickels (1942) and Miller and Stanley (1944) chiefly in the use of nearly parallel red (filtered) light. The optical path through the specimen was defined by 2 windows (each 8.0 mm wide by 4.0 mm high) cut in the front and back walls, respectively, of a brass tube into which the reaction tube was inserted. In the region above the windows, the brass tube was lined with a plastic sleeve, which served to minimize scratching of the reaction tube. The center of the light beam lay 9.0 mm above the bottom (outside) of the reaction tube, 16.0 mm below the meniscus (for 2 ml sample volume), and about 10.0 mm below the position of the sedimenting red blood cell boundary at the end of 1 hr. The transmission was measured with a photocell and galvanometer. Approximately 97 per cent of the optical density of a 1.0 per cent red blood cell suspension arose from scattering of the incident light.

Inhibitory activity. Inhibitors were characterized routinely as follows. Two

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series of 2-fold dilutions, related as 1:1.4, were prepared in bulk, and aliquots of 0.5 ml were transferred to reaction tubes. A volume of 0.5 ml heated virus, containing 3 to 4 hemagglutinating doses, was added to each tube, at the rate of 3 tubes per min, and mixing was completed by gentle stroking. After 30 min ± 20 sec, 1.0 ml of freshly prepared 2.0 per cent red blood cell suspension was added, with mixing as above. The transmission was read after an additional 60 min ± 20 sec. The inhibition titer (final dilution of inhibitor preparation at the conventional end point) was determined graphically (Hirst and Pickels, 1942) and standardized by reference to data obtained concurrently with a preserved egg white solution (EW Standard I, Lanni et al., 1950).

Modifications of this procedure are described in the experimental section. Each experiment included the preparation of a hemagglutination curve relating transmission to concentration of heated virus in the absence of inhibitor. This curve served both to specify the dosage of heated virus and to allow determination of the effective free virus concentration in virus inhibitor mixtures.

Precision. The precision of the measurements and the suitability of the overall technique were examined as follows. Ten replicate mixtures, each made with 1.0 ml 2 per cent red blood cell suspension and 1.0 ml heated virus, were set up at each of 5 different levels of virus dosage, covering a 16-fold range. Readings, made after 1 hr, were estimated to the nearest 0.1 per cent transmission. In no case did an individual reading differ from the average of its group by more than 3 per cent.

THEORY OF HEMAGGLUTINATION-INHIBITION

Preliminary considerations. The assumptions of the theory can best be understood by reference to the hemagglutination-inhibition data in figure 1. In this experiment (M63), inhibitor at various concentrations was allowed to react with a constant amount of heated virus for various periods before red blood cells were added (V-first mixtures). The data of the curve at the extreme left were obtained by mixing inhibitor and red blood cells and adding virus 5 min later (R-first mixtures). It was shown previously (Lanni and Beard, 1948b) and confirmed in the present study that the degree of inhibition in R-first mixtures is independent of the period of incubation preceding the addition of virus. This means that there is no significant interaction between inhibitor and red blood cells. On the other hand, the fact that inhibition increases with prolonged incubation of V-first mixtures prior to the addition of red blood cells points to a slow reaction between inhibitor and virus. Moreover, the V-first mixtures do not reach equilibrium in a 90-min period of preliminary incubation. Hemagglutination-inhibition will thus have to be dealt with as one or more kinetic processes.

The R-first data have especial analytical value since a period when red blood cells and inhibitor are present together and reacting with virus is a common feature of all mixtures. The interpretation of R-first data therefore precedes that of the more complex V-first experiments. The R-first data also allow division of the field of figure 1 at about I₀ = 2.0, so as to give a region on the right
where insignificant inhibition occurs in the presence of red blood cells, and a region on the left where part of the total inhibition can be attributed to reaction between inhibitor and virus occurring after red blood cells have been added.

Electron microscope observations (Heinmets, 1948; Dawson and Elford, 1949) indicate that several hundred virus particles can attach to a single cell. Estimates (see below) of the relative numerical concentrations of virus particles and red blood cells in the present experiments thus support the assumption that the total virus binding capacity of the red blood cells is greatly in excess of the total virus. Decreases in the effective red blood cell concentration, during reaction with virus, therefore can be neglected. A similar assumption can be made for the inhibitor in the gradient region of the R-first data since the inhibitor concentration here is many times the concentration capable of giving considerable inhibition in suitable V-first experiments. The experimental data do not justify extension of the assumption of inhibitor excess over the entire field of observations.

Theory. Consider the R-first situation. Let the symbols R, I, and V denote red blood cells, inhibitor, and virus, respectively, as well as their free concentrations at time t, and R₀, I₀, and V₀ their free concentrations at t = 0. R₀ can be conveniently expressed as red blood cells per ml. The concentrations of V
and I will be expressed as the number of their conventional units per ml. For \( V \), this unit is the hemagglutinating dose. For \( I \), the unit is defined as \( 1/6,400 \) of the inhibitor in 1.0 ml of a standard 10 per cent solution of egg white. Later an attempt will be made to evaluate these units in absolute numerical terms.

We assume that a given virus particle functions in hemagglutination by combining with receptors on two separate red blood cells according to the reactions:

\[
V + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} VR
\]

\[
VR + R \underset{k_6}{\overset{k_5}{\rightleftharpoons}} VR_2
\]

Other reactions such as:

\[
VR + V \rightarrow V_3R
\]

\[
V_3R + R \rightarrow V_2R_3
\]

\[
V_mR_n + V_xR_y \rightarrow V_{m+x}R_{n+y}
\]

may be expected to lead to the formation of increasingly large aggregates. Because of the spatial properties of \( R \) and \( V \), it seems unlikely that the reaction \( VR_2 + R \rightarrow VR_3 \) will occur to a significant extent. Lacking a detailed and verified theory of hemagglutination, we shall neglect the effect of the sizes of the complexes on the reactions between them.

Inhibitor is assumed capable of combining either with free virus or with virus attached to one red blood cell according to the reactions:

\[
V + I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} VI
\]

\[
VR + I \underset{k_8}{\overset{k_7}{\rightleftharpoons}} IVR
\]

As before, we neglect the effect of the sizes of \( V_mR_n \) complexes, and we let reaction (7) represent all the reactions between free inhibitor and once-attached virus. We now account for inhibition by excluding the reaction \( IVR + R \rightarrow IVR_2 \); i.e., we propose that combination of a given particle of virus with a single molecule of inhibitor is sufficient to neutralize that particle for hemagglutination, even though the particle may yet attach, or have already attached, to one cell. This assumption is reasonable since an estimate (Lanni et al., 1949) of the molecular ratio of inhibitor to virus at the conventional end point gave the value 3, at a time when the highest purification achieved for the inhibitor was 60-fold. The purification factor was later (Sharp et al., 1951) raised to 190, and the revised estimate of the \( I:V \) molecular ratio thus became unity (cf. Svedmyr, 1949b).

From the evidence of precipitation experiments (Lanni and Beard, 1948a;
Svedmyr, 1949b) and by analogy with virus-red blood cell reactions, it seems likely that reactions such as:

\[ \text{VI} + I \xrightarrow{k_9 / k_{10}} \text{VI}_2 \]  

(8)

also occur. Since VI is already regarded as inert for hemagglutination in the scheme formulated above, reaction (8) would have significance only through its effect in depleting I in mixtures prepared at low inhibitor concentrations.

Consider now the competition between R and I, first for free virus, V, then for once attached virus, VR. Let VR and VI represent the accumulated amounts of V which combine first with R and first with I, respectively. Assuming that reactions (1) and (6) are effectively irreversible in quiescent mixtures and that Ro and Io are each in substantial excess over Vo, then:

\[ \frac{d\overline{VR}}{dt} = k_1 VR_0 \]  

(9)

\[ \frac{d\overline{VI}}{dt} = k_3 VI_0 \]  

(10)

\[ \frac{d\overline{VR}}{d\overline{VI}} = \frac{k_1 R_0}{k_3 I_0} \]  

(11)

Integration gives:

\[ \frac{\overline{VR}}{\overline{VI}} = \frac{k_1 R_0}{k_3 I_0} \]  

(12)

When free V becomes negligible:

\[ V_0 = \overline{VI} + \overline{VR} \]  

(13)

\[ \overline{VR} = \frac{V_0}{1 + k_3 I_0 / k_1 R_0} \]  

(14)

R and I will compete for VR as it is formed, in accordance with reactions (2) and (7). Assuming that VR eventually becomes completely distributed between VR and IVR and proceeding as in the derivation of equation (14), we find:

\[ \overline{VR}_2 = \frac{\overline{VR}}{1 + k_7 I_0 / k_5 R_0} = \frac{V_0}{(1 + k_3 I_0 / k_1 R_0) (1 + k_7 I_0 / k_5 R_0)} \]  

(15)

Here VR2 represents the concentration of virus particles each of which is attached to two red blood cells; the other properties of the complexes (size, composition, etc.) remain unspecified. As an approximation, we assume that R and I compete with the same relative effectiveness for free as for once-attached V, i.e., \( k_3 / k_1 = k_7 / k_5 \), and obtain:

\[ \overline{VR}_2 = \frac{V_0}{(1 + k_3 I_0 / k_1 R_0)^2} \]  

(16)
This equation can be tested with R-first data if the degree of hemagglutination is taken to be directly determined by VR₂ and if VR₂ is itself equated with the amount of virus required to give the same effect in the absence of inhibitor. It seems unlikely that the last mentioned operation can be strictly correct since some of the virus of a virus-red blood cell mixture may reasonably persist as VR and not form VR₂. The estimates of VR₂ therefore will be in error by an amount equal to the quantity of virus which in appropriate virus-red blood cell mixtures is "wasted" as VR.

Equation (16) gave excellent fits with 4 independent sets of R-first data, with k₃/k₁ having the average value 2.2 × 10⁶; the individual values were 1.8, 1.9, 2.3, and 2.8 × 10⁶. In the calculation of these values, Rₙ was introduced as red blood cells per ml and Iₙ as inhibitor units per ml; the units of V are immaterial since they cancel out. In what follows, a factor f, defined by:

\[
f = \frac{N_i}{N_v} = \frac{\text{no. molecules inhibitor per unit \(N_v\)}}{\text{no. particles virus per hemagglutinating dose}}
\]

will be needed for the treatment of virus inhibitor reactions. Therefore it is convenient to convert k₃/k₁ to molecular (particle) concentration units by multiplying Iₙ by N_i. This gives k₃/k₁ = 2.2 × 10⁶/N_i, or k₃f/k₁ = 2.2 × 10⁶/N_v.

What would happen if the degree of hemagglutination were assumed to be determined not by VR₂ but by VR? In this case the appropriate equation would be (14), which, converted to a logarithmic form:

\[
\log \left( \frac{V_0}{VR} - 1 \right) = \log \frac{k_3}{k_1R_0} + \log I_0
\]

and tested by plotting the left-hand member against log I₀, yields straight lines with slopes of 1.16, 1.18, 1.19, and 1.22 in the 4 different R-first experiments. On the other hand, equation (16), in its corresponding logarithmic form, gives straight lines with slopes of 1.005, 0.996, 0.996, and 0.986 for the same experiments. Since the predicted slope is unity for each equation, we conclude that the mechanism requiring the formation of VR₂ is not only superior to the other, but very likely correct.

Turning to the V-first mixtures, our task is to find the values of V and I at the end of the period of preliminary incubation. VR₂ then can be obtained from equation (16). If both the consecutive reactions (6) and (8) are assumed to occur and to be effectively irreversible:

\[
\frac{dV}{dt} = -k_3fV \cdot I \tag{19}
\]

\[
\frac{dVI}{dt} = k_3fV \cdot I - k_5fVI \cdot I \tag{20}
\]

\[
\frac{d(fI)}{dt} = -k_3fV \cdot I - k_5fVI \cdot I \tag{21}
\]
Also:

\[ V_0 = V + VI + VI_2 \]  
\[ fI_0 = fI + VI + 2VI_2 \]  

Combining (19) and (20) and integrating gives:

\[ VI = 2.3V \log \frac{V_0}{V} ; \quad k_0 = k_3 \]  
\[ VI = \frac{V^{k_9/k_3}V_0^{l-k_9/k_3} - V}{1 - k_9/k_3} ; \quad k_9 \approx k_3 \]

Equation (19) may now be written:

\[ \frac{1}{k_3} = -\frac{V}{dV/dt} \left( fI_0 - 2V_0 + 2V + 2.3V \log \frac{V_0}{V} \right) ; \quad k_9 = k_3 \]  
\[ \frac{1}{k_3} = -\frac{V}{dV/dt} \left( fI_0 - 2V_0 + 2V + \frac{V^{k_9/k_3}V_0^{l-k_9/k_3} - V}{1 - k_9/k_3} \right) ; \quad k_9 \approx k_3 \]

For \( k_9/k_3 = 1/2 \), equation (27) gives:

\[ V^{1/2} = \frac{V_0^{1/2}(fI_0 - 2V_0)e^{-x}}{fI_0 - 2V_0e^{-x}} ; \quad x = k_3 \log \frac{fI_0 - 2V_0}{2} \]

For other values of \( k_9/k_3 \), there seems to be no useful solution, except that for \( k_9 = 0 \) (the case when reaction (8) is excluded) the integral is that of the differential rate equation for a bimolecular reaction:

\[ t = \frac{2.3}{k_3(fI_0 - V_0)} \log \frac{V_0(fI_0 - VI)}{fI_0(V_0 - VI)} \]

A more convenient form is:

\[ VI = \frac{fI_0 V_0(e^y - 1)}{fI_0 e^y - V_0} ; \quad y = k_3 \log (fI_0 - V_0) \]

from which, if \( f \) and \( k_3 \) are known, \( VI \) can be obtained and used for the calculation of \( V \) and \( fI \) at time \( t \) when red blood cells are added. \( VR_2 \) is obtained then from equation (16) by introducing \( V \) and \( fI \) for \( V_0 \) and \( fI_0 \), respectively, and taking into account the dilution which occurs when red blood cells are added.

Evaluation of the parameters. To evaluate \( f \), \( k_3 \), and \( k_1 \), we begin with a graphical treatment of the data at \( fI_0 = 1.5 \) in figure 1. At this concentration of inhibitor, negligible inhibition occurred in the R-first mixture; accordingly, it is assumed that in the V-first mixtures all of the effective reaction between inhibitor and virus occurred before red blood cells were added and thus that the hemagglutination reading indicates directly the concentration of free virus at time \( t \).

If the inhibitor were present in great molecular excess over the virus (\( V_0 = 4.0 \) hemagglutinating doses), a plot of \( \log V \) against \( t \) should be linear.
Such a plot showed pronounced curvature throughout, however, and a more complicated analysis therefore was needed.

Accordingly, $V$ was plotted against $t$, and values of $dV/dt$ at several intervals were obtained from tangents to the curve. These values were introduced in equation (27) (or equation (26) for $k_9 = k_3$). The resulting equations were solved simultaneously in pairs to give values of $f$ corresponding to values of $k_9/k_3$ taken from 0 to 1.0 in steps of 0.1. None of these values of $k_9/k_3$ yielded constant $f$ throughout the period of reaction; however, the extrapolated values of $f$ at $t = 0$ ranged from 1.92 to 2.08, with an average of 2.00. A similar procedure gave values of $k_3$ ranging from 1.11 to 1.17 $\times$ $10^{-2}$, with an average of $1.14 \times 10^{-2}$ cm$^3$ min$^{-1}$; division by $N_v$ converts this value to molecular units. Since the primary reaction $V + I \rightarrow VI$ is the only reasonable reaction occurring significantly in the early period, these values of $f$ and $k_3$ are likely to be correct, regardless of the nature of the secondary reactions and so long as the complex VI is inert for hemagglutination. The failure to discover the most likely value of $k_9/k_3$ is attributed for the present to the experimental error of the measurements. Reference to figure 1 at $I_0 = 1.5$ shows, however, that the data are fitted well by calculations for a simple bimolecular reaction ($k_9 = 0$).

A check on these values of $f$ and $k_3$, in terms of their product $k_9f = 2.28 \times 10^{-3}$, was obtained in two different ways. First, the values of $dV/dt$ employed above were plotted as their logarithms against $t$. The extrapolated value at $t = 0$, substituted in equation (19), gave $k_9f = 2.25 \times 10^{-3}$. A second check was obtained from the experimental data at $I_0 = 13.6$, $t = 3$ min, by assuming that inhibitor was in sufficient excess over virus at this point as to permit use of the pseudo-monomolecular reaction equation:

$$\frac{V}{V_0} = e^{-k_9fI_0t}$$

(31)

with $V$ being estimated from equation (16). The result was $k_9f = 2.9 \times 10^{-2}$.

For the experiment of figure 1, $k_9f/k_1$ was estimated in molecular units as $1.8 \times 10^6/N_v$ (see above). With $f = 2.00$ and $k_3 = \frac{1.14 \times 10^{-2}}{N_v}$ cm$^3$ min$^{-1}$:

$$k_1 = \frac{2.00 \times 1.14 \times 10^{-2}}{1.8 \times 10^6} = 1.3 \times 10^{-8} \text{ cm}^3 \text{ min}^{-1}.$$

In order to convert $k_3$ to molecular units, we need an estimate of $N_v$, the number of virus particles per hemagglutinating dose. Unpublished measurements of the hemagglutinative activity of several preparations of purified swine influenza virus gave the maximal value 50,000 hemagglutinating doses per mg nitrogen. With information (Sharp et al., 1945) on the physical properties of the (unheated) virus, this value yields $N_v = 4.0 \times 10^8$. The constant $k_3$, thus is estimated as $1.14 \times 10^{-2}/4.0 \times 10^8 = 2.9 \times 10^{-11}$ cm$^3$ min$^{-1}$. With $N_v = 4.0 \times 10^8$ and $f = 2.00$, the conventional unit of inhibitor is estimated to contain $8.0 \times 10^8$ molecules of inhibitor. Since $R_v$ is usually about $5 \times 10^9$ (red blood cells per ml)
and $V_0$ about $8 \times 10^8$ (particles per ml) and since the available evidence indicates that one cell can combine with several hundred virus particles, the assumption that $R_0$ is in effective excess of $V_0$ seems to have been reasonable (cf. Friedewald and Pickels, 1944).

**COMPARISON OF THEORY WITH EXPERIMENT**

*Experiment M63.* A volume of 0.5 ml heated virus, containing 8.0 hemagglutinating doses per ml, was added to 0.5 ml inhibitor (M60-PEI) at various concentrations. Several mixtures of each kind were prepared. After various periods at room temperature, 1.0 ml of 2.0 per cent red blood cell suspension was added to one mixture of each kind. For the R-first mixtures, red blood cells were mixed with inhibitor, and virus was added after 5 min. In all cases readings were made 1 hr after the 3 components were mixed. The data, shown in figure 1, have already been used for evaluation of the constants, as described above. As in the experiments which follow, $I_0$ is plotted as the total inhibitor units present; this is done to allow uniform presentation of R-first and V-first data.

The curves in figure 1 were calculated from equation (16), written as:

$$\overline{VR}_2 = \frac{V_0}{(1 + k_3 f N_v I_0/k_1 R_0)^5}$$  \hspace{1cm} (16')

and equations (22), (23), and (30), neglecting the formation of virus-inhibitor complexes other than VI. The parameters were $f = 2.00; N_v = 4.0 \times 10^8$; $k_1 = 1.3 \times 10^{-8} \text{ cm}^2 \text{ min}^{-1}; k_2 = 2.9 \times 10^{-11} \text{ cm}^3 \text{ min}^{-1}$. In dealing with V-first mixtures, the calculated values of $V$ and $I$, obtained from equations (22), (23), and (30) with VI set at 0, are entered as $V_0$ and $I_0$ in equation (16'), account being taken of the 2-fold dilution which occurs when red blood cells are added.

*Experiment M62.* This experiment tested the effect of variation in $V_0$. A volume of 0.5 ml virus, containing 2.0, 4.0, 8.0, or 16.0 hemagglutinating doses per ml, was added to 0.5 ml inhibitor (M60-PEI) at various concentrations. After 30 min, 1.0 ml of 2.0 per cent red blood cell suspension was added, and readings (figure 2) were made after 1 hr. For calculating the theoretical curves, the constants were given their values in M63.

*Experiment M102.* This experiment was concerned with the effect of variation in $R_0$. Virus (0.5 ml, 6.4 hemagglutinating doses per ml) was incubated with dilutions of inhibitor (0.5 ml, M37-PEII) for 30 min. Replicates of each mixture received 1.0 ml red blood cell suspension at various concentrations: 1.0, 1.5, 2.0, 3.0, or 4.0 per cent. Readings (figure 3) were made after 1 hr. Theoretical curves were calculated with the M63 values for the constants. Calculated $\overline{VR}_2$ values were referred to hemagglutination curves at the appropriate level of $R_0$ to give the theoretical transmission.

*Experiment M72.* Equation (16') and certain features of the underlying theory were here subjected to a more drastic test. Red blood cells (1.0 ml of 2.0 per cent suspension) were mixed with dilutions of inhibitor (0.5 ml, M37-PEII). Virus (0.5 ml, 7.6 hemagglutinating doses per ml) was added after 1 min
and readings were made after 1 hr. The sediments were immediately and thoroughly resuspended by gentle shaking, and readings were made again after 1 hr. The process was repeated once more. Control virus-red blood cell mixtures were treated similarly to provide a reference curve for each stage. The hemagglutination-inhibition data at the three stages are shown in figure 4.

The theoretical curve at the first stage was calculated from equation (16') with $k_3fN_v/k_1 = 2.3 \times 10^6$ (compare with $1.8 \times 10^6$ in the previous experiments). For calculating the second stage readings, the new assumption was made that resuspension caused all the red blood cells to become singly dispersed through the separation of each virus particle previously attached to two red blood cells from one and only one of these red blood cells. It was further assumed that there occurred no dissociation of virus-inhibitor bonds, or of virus-red blood cell bonds when the virus was attached to only one cell.

Inhibitor and red blood cells were now able to compete anew for freshly exposed virus surface. According to the theory, only one bond, either with I or with R, needed to be formed to decide the fate of a given virus particle. The second readings therefore were calculated according to the equation:

$$\bar{V}R_2'' = \frac{\bar{V}R_2'}{1 + k_3fN_vI_0/k_1R_0}$$

(32)
Figure 3. Hemagglutination-inhibition as a function of final red blood cell concentration for V-first mixtures incubated preliminarily for 30 min (Experiment M108). Theoretical curves. HD = hemagglutinating doses.

Figure 4. Effect of resuspension on hemagglutination-inhibition in R-first mixtures (Experiment M12). Successive readings (top to bottom) have been fitted (solid lines) by an extension of the theory. For the meaning of the dashed line, see text.
in which the superscripts number the stages. The third readings were calculated from:

$$\overline{VR}_3''' = \frac{\overline{VR}_3''}{1 + k_2 f N_v I_0 / k_1 R_0}$$

(33)

The value of $k_2 f N_v / k_1$ was kept constant at $2.3 \times 10^4$. As seen in figure 4, the theoretical curves for the three stages fit the data as well as may be expected.

![Figure 5. Transmission as a function of time of reading for virus-red blood cell mixtures (curves 1, 4, and 6), inhibited mixtures of the V-first type (curves 2, 3, and 5), and a red blood cell-saline control (curve 7) (Experiment M75).](image)

This agreement supports the assumption (see above) that $k_7 / k_8 = k_3 / k_1$ since, except for the rupture of certain V—R bonds, only reactions involving $k_6$ and $k_7$ have been postulated to account for the increase in inhibition which follows resuspension. In addition, the experimental results demonstrate the failure of R-first mixtures to reach equilibrium rapidly.

A complication in these studies arises from the fact that the reference hemagglutination curves likewise tend to fall with resuspension. The effect which this phenomenon would have, if it were the only contributor, was evaluated for the third stage readings by referring the calculated first stage $\overline{VR}_3$ values directly...
to the third stage hemagglutination curve. From the result, shown as the dashed line in figure 4, it is clear that only part of the total drop can be accounted for in this way.

Similar resuspension experiments were carried out with V-first mixtures incubated preliminarily for 3, 10, 30, or 90 min. A definite, though small, increase in inhibition was observed with the 3-min set following resuspension; but when preliminary incubation was for 10 min or longer, the observed decrease in transmission was identical with that occurring in virus-red blood cell controls. The explanation of these results is that in partially inhibited mixtures the inhibition can increase with resuspension only when I₀ is sufficiently great as to allow effective competition of I with R. Reference to figure 1 shows that this condition is not fulfilled in the case of virus inhibitor mixtures incubated preliminarily for 10 min or longer.

Experiment M73. The calculation of \( \overline{VR}_2 \) from reference hemagglutination curves involves the assumption that estimates of \( \overline{VR}_2 \) are independent of the time of reading, so long as the transmission of inhibited and uninhibited mixtures is determined after the same interval. This assumption was tested by making periodic readings on several inhibited mixtures of the V-first type (preliminary incubation for 15 min) and on several virus-red blood cell mixtures. The resulting curves of log transmission against time of reading are shown in figure 5. For each value of I₀, estimates of \( \overline{VR}_2 \) were obtained in the period from 30 to 84 minutes by reference to the appropriate hemagglutination curves. The results were: for I₀ = 0.75, \( \overline{VR}_2 = 3.34 \) (avg) ± 0.06 (S.D.); for I₀ = 1.5, \( \overline{VR}_2 = 2.63 \) ± 0.04; for I₀ = 3.0, \( \overline{VR}_2 = 1.55 \) ± 0.04. No chronological correlation was evident.

**DISCUSSION**

The quantitative theory presented above explains with considerable precision the results of detailed experiments on hemagglutination-inhibition. The theory involves so many assumptions, however, that it seems desirable to consider whether any independent support can be adduced. Hirst (1942) has reported experiments on the rate of adsorption of (unheated) influenza virus B (Lee strain) onto chicken red blood cells. One volume of Lee virus in allantoic fluid was mixed with one volume of 3 per cent red blood cell suspension at 23 C; supernatants, obtained by centrifuging after various periods, were assayed for residual hemagglutinative activity. Estimating R₀ as \( 7.5 \times 10^7 \) and employing the equation:

\[
V = V_0 e^{-k_1 R_0 t}
\]  
(34)

we find that at \( t = 1, 2, \) and 3 min, \( k_1 = 1.33, 1.38, \) and \( 1.26 \times 10^{-8} \) cm² min⁻¹, respectively, with an average of \( 1.32 \times 10^{-8} \), for the early period of the adsorption phase of the complex adsorption-elution cycle. This result may be compared with our value \( 1.3 \times 10^{-8} \), obtained for heated swine influenza virus through the estimation of \( k_f/k_1, k_3, \) and \( f \).

An alternative estimate of \( k_1 \) can be obtained from the Schlesinger-Delbrück equation which has recently received successful application in studies of the
adsorption of bacteriophage onto its sensitive host (Puck et al., 1951). The equation, which estimates the upper limit of the rate constant if all collisions result in combination, is:

\[ k_{\text{max}} = 4\pi Dr \]  

(35)

where \( r \) is the mean radius (cm) of the adsorbing particle (red blood cells in our experiments) and \( D \) is the diffusion constant \((\text{cm}^2 \text{ sec}^{-1})\) of the virus. \( D \) for swine influenza virus does not seem to have been measured, but may be estimated from the Einstein-Sutherland equation:

\[ D = \frac{RT}{6\pi \eta N} \]  

(36)

as about \( \frac{8.31 \times 10^2 \times 298}{6\pi \times 5.85 \times 10^{-6} \times 0.01 \times 6.02 \times 10^2} = 3.7 \times 10^{-8} \text{ cm}^2 \text{ sec}^{-1} \) at

25 C. Here \( R \) is the gas constant, \( T \) the absolute temperature, \( r \) the radius of the hydrated virus particle (Sharp et al., 1945), \( \eta \) the viscosity of the medium, and \( N \) the Avogadro number. The chicken erythrocyte, with estimated length, width, and thickness of 13, 7, and \( 5 \times 10^{-4} \text{ cm} \), respectively, has a mean radius of about \( 4.4 \times 10^{-4} \text{ cm} \), calculated as the radius of a sphere of equal volume. We find, therefore, that:

\[ k_{\text{max}} = 4\pi \times 3.7 \times 10^{-8} \times 4.4 \times 10^{-4} \times 60 
\[ = 1.2 \times 10^{-8} \text{ cm}^2 \text{ min}^{-1}. \]

With due respect for the approximations involved, this excellent agreement, beyond confirming that an erythrocyte does indeed possess numerous receptors for virus, suggests that the virus-red blood cell reaction is characterized by nearly 100 per cent collision efficiency. As discussed by Puck et al. (1951) for bacteriophage, such an agreement indicates that the activation energy must be very low and, therefore, that the interaction is likely to be predominantly ionic. This conclusion in turn suggests that the reaction would be found (a) to have a low temperature coefficient and (b) to be highly sensitive to salt concentration.

These expectations are confirmed by published observations. Hirst (1942) found that the rate of adsorption of PR8 virus onto chicken red blood cells was relatively insensitive to temperature between 4 and 37 C, in contrast with the marked temperature dependence which characterized the elution (enzymatic) phase of the adsorption-elution sequence. Other workers (Lowell and Buckingham, 1948; Davenport and Horsfall, 1948; Flick et al., 1949) have shown that influenza viruses fail to combine with red blood cells in the absence of salt. The combination of pneumonia virus of mice with lung particles and red blood cells is likewise inhibited at low ionic strength (Davenport and Horsfall, 1948); however, the GDVII strain of mouse encephalomyelitis virus appears capable

\[ \text{The increase in adsorption rate of heated Lee virus with temperature, reported by Dawson and Elford (1949), is not greater than one would expect from a decrease in the viscosity of the medium.} \]
of combining with red blood cells under these conditions although hemagglutination is prevented (Fastier, 1951). Indeed, the literature of virus hemagglutination is replete with reports of the significant role of external ions in this and related phenomena. These observations emphasize a basic similarity between the initial stages, at least, of virus-red blood cell and phage-bacterium interactions.

Much less is known about the relevant characteristics of virus inhibitor interaction. For our present purpose, it seems desirable to estimate the maximal value of \( k_3 \) (the rate constant for virus inhibitor combination) by resorting to equation (35). The calculation requires an estimate of the molecular weight of the inhibitor, for which physical data (Lanni et al., 1949) have indicated the minimal value 7.6 \( \times \) 10\(^3\). An alternative estimate can be based on the value of \( N_1 \), the number of inhibitor molecules comprising the conventional unit; this number was found to be 8.0 \( \times \) 10\(^8\) (see above). From purification data it can be calculated that one unit of inhibitor (in preparations with purification factor 190) contains 1.32 \( \times \) 10\(^{-9}\) g nitrogen or 1.06 \( \times \) 10\(^{-8}\) g protein. The anhydrous molecular weight therefore is computed as:

\[
\text{mol wt} = \frac{6.02 \times 10^{23} \times 1.06 \times 10^{-8}}{8.0 \times 10^8} = 8.0 \times 10^6.
\]

Assuming 33 per cent hydration and 1.25 wet density, the hydrated molecular weight is 1.07 \( \times \) 10\(^9\), and the radius and diffusion constant \( (D_0) \) of an equivalent sphere are 1.5 \( \times \) 10\(^{-8}\) cm and 1.5 \( \times \) 10\(^{-7}\) cm\(^2\) sec\(^{-1}\), respectively. Correction for the asymmetry of the inhibitor molecule, estimated (Lanni et al., 1949) as 90:1, gives for a rigid prolate ellipsoid of revolution \( D_o/D = 3.9 \) (Cohn and Edsall, 1943). \( D \) is therefore about 3.8 \( \times \) 10\(^{-8}\) cm\(^2\) sec\(^{-1}\). The maximal value of \( k_3 \) is estimated then as:

\[
k_{\text{max}} = 4\pi \times 3.8 \times 10^{-8} \times 5.8 \times 10^{-8} \times 60 = 1.7 \times 10^{-10} \text{ cm}^3 \text{ min}^{-1}.
\]

This value is clearly a gross approximation,\(^4\) but the reasonable agreement with the experimental value 2.9 \( \times \) 10\(^{-11}\) indicates that virus-inhibitor interaction, just as virus-red blood cell interaction, is likely to be characterized by high collision efficiency and to be predominantly ionic.\(^5\) In addition, the high molecular

\(^4\) The estimate is not greatly sensitive to the values taken for hydration, wet density, and asymmetry, and a similar result can be calculated for an oblate ellipsoid model. In the application of equation (35) to virus inhibitor interaction, a small error is introduced by neglect of the collision cross-section of the inhibitor molecule. Use of the more exact relation (Moelwyn-Hughes, 1947, p. 245):

\[
k_{\text{max}} = 2\pi(D_1 + D_r)(r_1 + r_r)\left[1 + \frac{(r_1 + r_r)(D_1^{1/3} + D_r^{1/3})}{\pi^{1/3}(D_1 + D_r)}\right]
\]

gives 2.1 \( \times \) 10\(^{-10}\) cm\(^3\) min\(^{-1}\) as the maximal estimate of \( k_3 \). With \( D_r \) taken as 5 \( \times \) 10\(^{-10}\) cm\(^2\) sec\(^{-1}\), \( k_1 \) is estimated as 1.5 \( \times \) 10\(^{-9}\) cm\(^3\) min\(^{-1}\).

weight of the inhibitor, together with its extended molecular configuration, lends credibility to our basic postulate that one molecule of inhibitor, attached to a virus particle, is sufficient to neutralize that particle for hemagglutination.

These encouraging results underline the need for new and critical experiments not only on the processes of hemagglutination and hemagglutination-inhibition but also on the relevant physical and chemical properties of the interacting agents. The present theory is not likely to survive unchanged. It seems clear, however, that some such theory will be needed for the detailed interpretation of the more complex interactions involving virus as enzyme.

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We are grateful to associates at the University of Illinois and elsewhere for their interest and counsel.

SUMMARY

A quantitative theory of hemagglutination-inhibition has been developed from the following basic postulates:

1. A given virus particle contributes to hemagglutination only by combining with receptors on two separate red blood cells.

2. Attachment of a single inhibitor molecule to a virus particle suffices to neutralize that particle for hemagglutination, even though the particle may yet attach to a single cell.

3. The reaction of virus (V) with inhibitor (I) or red blood cells (R) is effectively irreversible in quiescent mixtures and does not reach completion (or equilibrium) in ordinary experimental periods. The reactions therefore are handled as rate processes.

The parameters of the theory were evaluated from a restricted set of hemagglutination-inhibition data obtained with heated swine influenza virus, egg white inhibitor, and chicken red blood cells. The theory was then applied successfully to the interpretation of a variety of hemagglutination-inhibition data.

Comparison of the experimental rate constants for the two primary reactions \(V + R \rightarrow VR; V + I \rightarrow VI\) with maximal theoretical estimates indicated that both reactions are characterized by remarkably high collision efficiency.

The significance of this result is discussed in relation to the probable mechanism of the interactions. A by-product of the analysis was an estimate of the molecular weight of the inhibitor, given as \(8 \times 10^4\).

Several predictions of the theory were verified by reference to already published observations, which had not previously been properly interpreted or integrated.

REFERENCES


SHARP, D. G., TAYLOR, A. R., MCLEAN, I. W., JR., BEARD, D., AND BEARD, J. W. 1945 Density and sizes of the influenza viruses A (PR8 strain) and B (Lee strain) and the swine influenza virus. J. Biol. Chem., 159, 29-44.


replicate plates incubated aerobically failed to yield lecithinase producing bacteria. Twenty-nine lecithinase positive isolates were obtained from the former plates and all produced a stormy fermentation in reduced iron-milk and none would grow aerobically. Known cultures of *Clostridium perfringens* and *C. sporogenes* grew and produced lecithinase in the modified medium under anaerobic conditions while *B. cereus* and several unidentified aerobic sporeformers (isolated from feces), which split lecithin under either aerobic or anaerobic conditions, failed to grow in the azide containing medium. After many trials, *Bacillus* spp have not been isolated from plates inoculated with fecal suspensions and poured with the azide-egg yolk agar medium, even though they were encountered in rat feces in greater numbers than lecithinase producing clostridia.

Plate counts of lecithinase producing anaerobes in this medium have been carried out at 37 C for 24 hours in an atmosphere of 90 per cent H₂ and 10 per cent CO₂. No more than 20 lecithinase positive colonies could be distinguished clearly in a single petri plate (the diameter of the zone of precipitate varied from 10 to 30 mm). Incubation for more than 24 hours resulted in even larger zones.

It is thought that the application of this method by others for the isolation of *C. perfringens* and *C. sporogenes* from a variety of natural materials, e.g., soil, pus, and certain food products, might be as successful as it was found in this laboratory for the study of the anaerobic bacteria in the rat's intestine. Future reports will include details of the results obtained with this azide-egg yolk medium.

**ERRATUM**

A QUANTITATIVE THEORY OF INFLUENZA VIRUS HEMAGGLUTINATION-INHIBITION

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An error appears in the recently published article with the above title (J. Bact., 64, 865, 1952). Figures 2 and 3 have been interchanged, and the last statements of the legends for these figures have also been interchanged.

The figure appearing on p. 875 should have the following legend:

*Figure 3.* Hemagglutination-inhibition as a function of final red blood cell concentration for V-first mixtures incubated preliminarily for 30 min (*Experiment M108*). Theoretical curves. RBC = red blood cells.

The legend for the upper figure on p. 876 should read:

*Figure 2.* Hemagglutination-inhibition as a function of total virus concentration for V-first mixtures incubated preliminarily for 30 min (*Experiment M68*). Theoretical curves. HD = hemagglutinating doses.