

QUANTITATIVE ASPECTS OF THE SPONTANEOUS ELUTION OF INFLUENZA VIRUS FROM RED CELLS*

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Influenza virus attached to chicken red cells at 37°C. is eventually eluted into the medium. The virus appears to be unchanged as a result of this experience, but the red cells are thereafter unable to adsorb new virus (1). Although much attention has been devoted to this reaction, and some of its enzymatic aspects elucidated, study of the mechanism has been hindered by lack of a simple, accurate method for measuring absolute numbers of virus particles capable of hemagglutination. With the development of such a method (2) the kinetics of the virus-cell interaction has become susceptible to more quantitative study, some experiments of which are herewith presented.

Methods and Materials

The preparation of the virus and red cells and the method of titrating absolute numbers of hemagglutinating virus particles have been described elsewhere (2). The number of hemagglutinating particles per cm.³ was determined in each virus stock, measured aliquots of which were used for individual experiments. Relative determinations of virus content within each experiment were then carried out by the pattern technique (3) using a concentration of 7.2×10^6 red cells/cm.³ in each tube. Serial-doubling dilutions were employed in experiments in which relatively less accuracy was needed; in others the following series of virus dilutions was used: 1:2¹, 1:2^{1.25}, 1:2^{1.59}; 1:2², 1:2^{2.25}, 1:2^{2.59}; 1:2³, 1:2^{3.25}, 1:2^{3.59}, etc. Under these conditions the amount of virus in the last dilution tube to give a positive pattern is 3.5×10^6 hemagglutinating particles/cm.³ (2). All experiments were carried out in 0.9 per cent NaCl adjusted to pH 7 with 10⁻³ M phosphate buffer, hereafter called "attachment medium."

EXPERIMENTAL RESULTS

Maximum Number of Virus Attachment Sites per Red Cell

The maximum number of virus particles capable, on the average, of attaching to a single chicken red cell was determined by the following experiment.

To a series of tubes at 5°C. containing red blood cells in a final concentration of 2.8×10^7 /cc., varying amounts of virus were added so that final virus/cell ratios (v/n) of 25:1, 250:1, and 500:1, respectively, were obtained. After a 30 minute attachment period, the

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tubes were centrifuged and the supernatants carefully removed and assayed for residual, unattached virus. The sedimented cells, still at 5°C., were washed once in attachment medium, an operation which does not remove any virus already attached to cells, and a second virus input of fifty particles per red cell then added. After a second 30 minute period, the reaction tubes were again centrifuged and these supernatants also assayed for residual unattached virus. Table I shows the results of such an experiment.

It is evident that cells which in the primary adsorption period attached 25 virus particles still have a high avidity for the virus; cells which have adsorbed 250 particles still can attach most, but not quite all, of an additional inoculum of 50 virus particles. Those attaching approximately 300 can no longer attach any measurable amount of influenza virus. Hence, the maximum number of virus-attaching sites per cell appears to lie in the neighborhood of 300.

TABLE I
Determination of the Total Number of Virus Attachment Sites on Chicken Red Cells

<i>v/n</i> ratio in 1st attachment tube	Amount of virus attached in 30 min.	<i>v/n</i> added for 2nd attachment cycle	Amount of attachment of second virus input	Maximum No. of virus particles capable of attachment by each cell
	<i>per cent</i>		<i>per cent</i>	
(A) 25:1	100	50:1	100	>75
(B) 250:1	100	50:1	98	299
(C) 500:1	67	50:1	0	329

Virus was added in the multiplicities indicated to each of three tubes containing 2.8×10^7 cc. red cells at 5°C. After a 30 minute attachment period the supernatants were thoroughly decanted, their virus titers determined, and the cells washed in saline to remove any unattached virus, and then an additional virus inoculum corresponding to 50 particles per cell was added to each tube. The amount of this second virus inoculum which was taken up by the cells was determined.

Since the cross-sectional area of influenza virus is approximately 1.0×10^6 square Angstrom units, (4) while the total area of the chick red cell is approximately 1.4×10^{10} A² (5), 300 virus particles would only occupy approximately 2 per cent of the total surface. Thus, only a small fraction of the red cell surface is actually available for attachment of influenza virus.

By a similar procedure the number of PR8 attachment sites on human type O red blood cells was found to be approximately 250 particles, a value again representing approximately 1.8 per cent of the total surface of the cell.

The rate of attachment of PR8 to chicken red cells was determined by the standard procedure previously described (6). Linearly logarithmic curves were obtained from which a value of the attachment constant, K ,¹ was calculated to be 6.6×10^{-9} cm.³ min.⁻¹ This constant which is practically independent

¹ Defined by the equation $2.3 \log V_t/V_0 = Knt$, in which V_t = concentration of free virus remaining at time t ; V_0 = initial virus concentration, and n = No. of red cells/cm³.

of temperature between 0 and 37° remained virtually unchanged for cell concentrations ranging from 1.25×10^7 to $7.5 \times 10^7/\text{cm.}^3$

Demonstration That the Virus Initiates a Spreading Disturbance on the Cell Membrane

Only a small fraction of the cellular attachment sites needs to be occupied by a virus in order for the elution reaction to occur, as shown in the following experiment.

To two tubes, each containing 8.7×10^7 red cells/cm.³, influenza virus was added in a virus:cell multiplicity of 40:1. After 15 minutes at 0°C., all of the virus in both tubes had become attached to cells. That the cells were far from saturated was demonstrated by adding to one tube an additional virus inoculum, corresponding to a multiplicity of 100:1, all of which became attached to the cells in 15 minutes. The second tube, still containing only 40 virus particles per cell, was placed in a 37°C. bath for 6 hours. At the end of this time 75 per cent of the original virus inoculum had been eluted into the supernatant fluid. It may be concluded that the action of each virus particle is not confined to a single attachment site on the cell surface, but rather, is responsible for the destruction of a much larger area.

Such an action would appear to involve one of two mechanisms: (a) each virus particle initiates at its point of attachment a migrating disturbance which can lead to destruction of receptors at distant points, or (b) each particle destroys the cell receptor at its point of attachment only, and is then eluted into the medium from which it is reattached to another site so quickly that no appreciable quantity of free virus can be detected until most of the cellular receptors are destroyed. Although both possibilities have previously been considered (7, 8), no experimental evidence seems to exist which would distinguish between them. Hence, the test described in Table II was performed in which reattachment of any eluted virus was prevented by a hundredfold dilution of the reaction tube. After such dilution the resulting cell concentration (2.8×10^5 cells/cm.³) is one which experiment has shown to produce no measureable virus attachment in 3 hours. The data of Table II show clearly that loss of receptor sites still proceeded efficiently in this tube. Thus, an amount of virus equivalent to 8 per cent of the cell's actual attachment capacity has inactivated all of the receptor sites, under conditions in which repeated cycles of attachment were precluded. The conclusion can be drawn that, as a result of a single act of attachment, each virus particle induces a travelling change in the cell wall that involves an area far exceeding that represented by the cross-section of virus.

Receptor Destruction Precedes Elution

Data have been presented showing that an amount of virus equivalent to a small fraction of the cell's attachment capacity can destroy practically all

TABLE II

Demonstration That a Single Act of Virus Attachment Initiates a Spreading Disturbance on the Cell Surface

Cells which have attached an average of 19 virus particles lose practically all of their 300 attachment sites, and also elute a considerable portion of their attached virus even under conditions in which cyclical attachment and elution are prevented.

Influenza virus was added in a multiplicity of 19:1 to a suspension of red cells ($2.8 \times 10^7/cm.^3$) at $0^\circ C$. After a 10 minute period at this temperature during which 98 per cent of the virus was attached, the reaction mixture was divided in half. One part was diluted 1:100 in saline, then incubated at $37^\circ C$. for 3 hours, while the other was similarly incubated without dilution. Then both tubes were centrifuged at $0^\circ C$. and the supernatants tested for virus elution. The centrifuged cells from both tubes were then collected, washed, and resuspended in a volume of new saline, such that both sets of cells were now restored to the original concentration of $2.5 \times 10^7/cm.^3$ Both cell suspensions were then tested for loss of receptor sites by measuring their ability to attach a new virus inoculum, representing a v/n ratio of 2:1.

Amount of initially adsorbed virus eluted after 3 hrs. at $37^\circ C$.		Amount of new virus inoculum attaching to cells at $0^\circ C$. after elution of the original virus		
1:100 diluted cell suspension	Undiluted suspension	Attachment to test cells which have eluted virus		Attachment to fresh cells at same concentration (control)
		Diluted and re-concentrated cells	Undiluted cells	
<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
52	56	12	0	95

Other control experiments demonstrated that the cell concentration in the diluted tube ($2.8 \times 10^5/cc.$) is so small as to cause no appreciable virus attachment in 3 hours. Hence, if virus had eluted from these cells without destroying additional receptor sites, the cells should have demonstrated unimpaired ability to take up the new virus inoculum.

TABLE III

Demonstration that a small amount of virus, equivalent to 1 per cent of the cellular attachment sites (0.02 per cent of the cell area), can cause inactivation of all the attachment sites on the red cell before any appreciable elution has occurred.

Duration of incubation at $37^\circ C$.	(A)	(B)
	Amount of attached virus eluted	Uptake of a challenge virus inoculum
<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>
0	0	100
12	2	0

PR8 was added to red cells in an average multiplicity of 3:1 at $0^\circ C$. Attachment was complete. The cells were then incubated at $37^\circ C$. Aliquots were removed at various times and tested (A) for elution of the original virus inoculum, and (B) for the number of attachment sites inactivated as judged by ability of the cells to bind a new virus inoculum of 10 particles per cell.

of the cell's receptors. The experiment described in Table III demonstrates that this destruction is complete before an appreciable amount of the virus has been eluted from the cells. As is also evident from comparison of the data

in parts A and B of Table IV, when virus multiplicities large enough to produce both receptor destruction and extensive virus elution are employed, receptor site destruction precedes virus elution over the entire range of multiplicities employed.

The Requirement of the Elution Reaction for Multiple Virus Attachment

Hanig (7) reported that elution of influenza virus from red cells increases with the amount of virus added. We have studied the effect of varying the

TABLE IV

(A) *Effect of Virus Multiplicity on Spontaneous Elution at 37°C.*

The procedure of Table II was repeated with a large range of virus-cell multiplicities.

Average v/n	Amount of attached virus eluted at following times (in hrs.)				
	2	4	6	12	24
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	0	0	0	0	0-2
3	0	7	3	2	13
9	2	16	16	10	12
20	16	16	16	20	66
90	13	32	63	48	74
150			80		

(B) *Effect of Virus Multiplicity on Destruction of Attachment Sites*

Average v/n	Amount of attachment of a second virus inoculum added to cells at following times (in hrs.)				
	2	4	6	12	24
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	100	97	95	94	91
3	94	75	50	0	0
9	0	0	0	0	0
20	0	0	0	0	0
90	0	0	0	0	0

virus:cell ratio by carrying out the procedure of Table III on a series of tubes with constant cell concentration, but varying amounts of virus. The results of these experiments, summarized in Table IV A, show a strong dependence of the elution on the multiplicity. The effect on the receptor sites is parallel, but appears earlier in time, as previously noted (Table IV B).

The data suggest an apparent threshold in the number of virus particles necessary to initiate the receptor destruction and virus elution reactions within 24 hours. Exact determination of the threshold value is not possible from these data, however, because the multiplicities given are only average values, each tube actually containing a Poisson distribution of cells with varying numbers

of viruses. Thus, cells containing a number of virus particles greater than the threshold value will eventually elute, freeing virus for reattachment to other cells, some of which could in this way also gain the threshold value. Hence, a cascading sequence of virus elution undoubtedly occurs in some of these tubes, producing a complex situation for quantitative analysis.

The data of Table IV permit the following conclusions: (a) A single virus particle attached to a red cell appears insufficient to evoke receptor site destruction and virus elution within 24 hours. The small amount of reaction which is evident when the average v/n is unity, is undoubtedly due to those cells containing several virus particles, which are present in such a population. Calculation shows that 26 per cent of the cells will have acquired 2 or more virus particles, and 8 per cent will possess 3 or more. Since, at the most, 2 per cent of the virus is eluted at 24 hours, the threshold appears to be greater than 2 viruses per cell. Verification of this conclusion is presented in an experiment described below.

(b) The demonstration that an average multiplicity of three causes complete destruction of receptor sites in more than 6 but less than 12 hours appears to fix the threshold in the neighborhood of 3. Since an average multiplicity of three produces a distribution in which appreciable numbers of cells have 5, 6, or 7 virus particles, and since on elution these could reattach to cells which had not yet reached the threshold value, the critical number is still uncertain. An upper limit for the threshold value can be estimated by the following considerations: An average multiplicity of three suffices to destroy attachment sites completely on all the cells. If the actual threshold were as high as 7, for example, the number of cells with this multiplicity could be calculated by the Poisson formula. If all these eventually eluted their virus completely, the virus would presumably be randomly distributed among the remaining cells. The per cent of these which would then acquire 7 viruses can be calculated. These may be considered to elute, and the resulting distribution again computed. Analysis of the effects of all such possible cycles reveals that for an average multiplicity of three, a threshold requirement of 7 could not result in receptor destruction on all of the cells, as was observed experimentally. Such calculations applied to this data appear to set the limits of the threshold value for site destruction and virus elution (assuming the second to follow automatically if the first is complete) between 3 and 5 virus particles per cell.

Since several virus particles are required to initiate the irreversible changes associated with spontaneous elution, it becomes necessary to consider whether only a fraction of the hemagglutinating virus population is actually active in the latter process. To test this point, an experiment was designed in which a negligibly small fraction of the red cells received more than a single virus particle, and in which repeated cycles of virus elution and reattachment to other cells was precluded by large scale dilution of the mixture before placing

it in the 37°C. bath for the elution step. After varying periods of incubation, the supernatants were tested for virus elution. An average virus:cell multiplicity of 0.17 was employed. Because of this low virus input, and the large subsequent dilution, egg infectivity was employed to test for virus elution. A typical set of experimental data is presented in Table V. They indicate that no elution was detectable after incubation for as long as 24 hours. Control tests demonstrated that the assay method employed can easily detect as little as 50 virus particles. Hence, the supernatants contained less than 500 particles/cm.³. A final virus concentration of 10⁵/cm.³ would have represented 100 per

TABLE V

Test of Elution of PR8 from rbc under Conditions in Which the Actual Multiplicity Is 1:1

To 1 per cent rbc (5.8×10^7 /cc.), 1×10^7 PR8/cc. was added and the mixture incubated 30 minutes at 5°C. to give maximum attachment with no elution. Aliquots were diluted 1:100 in buffered saline to stop adsorption, and then transferred to a 37°C. bath. The supernatant was sampled at 12 and 24 hours. These samples were inoculated into 9 to 10 day chick embryos which were incubated 48 hours at 35°C. and checked for development of hemagglutinins. A control tube was employed to which no cells were added but in which the virus was diluted and otherwise treated in an identical manner including the 37°C. incubation, as in the test. 0.1 cc. samples of 10⁻², 10⁻¹, and undiluted supernatants were inoculated into embryonated eggs, 3 of which were employed in each dilution. After 48 hours of incubation at 35°C. the allantoic fluids were tested for presence of hemagglutinins. The table indicates the presence (+) or absence (-) of hemagglutinins in each egg inoculated.

Supernatant dilution inoculated	<i>Elution of PR8 virus from rbc in low v/n ratios</i>					
	12 hr. elution			24 hr. elution		
	Undiluted	1:10	1:100	Undiluted	1:10	1:100
Test	---	---	---	---	---	---
Control: tube containing same amount of virus but with no cells.	+++	+++	+++	+++	+++	+++

cent elution. Hence, the extent of elution was less than $\frac{1}{2}$ per cent. If 1 in 3 virus particles had been active in elution, the supernatant titer should have corresponded to 33 per cent elution. It may therefore be concluded that the necessity for multiple virus attachment in order to evoke elution within a period of 24 hours, is not due to incompetence of the majority of the virus population with respect to this reaction.

This experiment, while demonstrating that singly infected red cells do not elute spontaneously under the prescribed conditions, also indicates that the threshold requirement is probably greater than 2. For with the multiplicity of 0.17, which was employed, 1.2 per cent of the cells would have received 2 or

more virus particles. If these had eluted their bound virus, the concentration in the supernatant would have been $1.4 \times 10^4/\text{cm.}^3$ which also should have been detectable by the assay method employed.

It was not possible to extend these tests to periods greater than 24 hours because of the onset of hemolysis of the red cells.

DISCUSSION

Hanig (7), reasoning from the shapes of electrophoretic mobility curves of red cells to which varying numbers of influenza virus particles had attached, also arrived at approximately 300 virus particles as the saturation value for human red cells. However, the reliability of his figure was limited because of uncertainties in the method used to assay absolute numbers of hemagglutinating particles, and the existence of unproved assumptions in the calculations employed.

The data here presented offer interesting points of comparison to the interaction of the coli bacteriophages of the T-system with their host cells. These cells have so high a density of receptor sites on their surface that the number of virus particles capable of attachment is equal to the number that can fit in the given space in close packed array (9). Hence, although the red cell surface is approximately 25 times larger than *Escherichia coli*, and influenza virus is even slightly smaller than T2 bacteriophage, the saturation value of *E. coli* for this phage is approximately the same as that of the red cell for influenza virus. The similarity of the virus-cell attachment constants of the two systems ($6 \times 10^{-9} \text{ cm.}^3 \text{ min.}^{-1}$ for the influenza and red cells, as compared to $4.0 \times 10^{-9} \text{ cm.}^3 \text{ min.}^{-1}$ for bacteriophage (6)) confirms the fact that for all its vastly greater size, the red cell has only as many virus attachment sites as a bacterial cell. Moreover, since in the latter case it was shown that virus-cell attachment proceeds approximately as fast as the collision rate of the two bodies (6) it can be concluded that in the PR8-red cell system, the rate of attachment is equal to the collision rate of a virus particle with an attachment site. As has been discussed (6), this relationship is strong evidence for the electrostatic nature of the bond formation which constitutes attachment.

The striking difference in the per cent of cell surface areas occupied by virus attachment sites in the bacterial and avian-mammalian systems respectively would appear to be a fundamental difference between the two systems. Experimental data indicate that animal virus host cells are comparable to the red cell in their limited number of attachment sites (10). It is possible that this difference also underlies another important difference in behavior of the two systems: Animal viruses are liberated from their infected host cells in a gradual process extending over many hours (11, 12); whereas, in the T system bacteriophages, a cell discharges all of its virus simultaneously and is lysed in the process. Evidence has been presented in the bacterial system that each virus attach-

ment site offers the opportunity of triggering a lytic enzyme in the cell wall, which presumably permits entrance of the virus DNA (13). If the number of attachment sites on a cell surface is indeed also a measure of the number of potential lytic sites, it is understandable why lysis involves the entire surface of a bacterial cell; whereas, in the animal cell, if only a small fraction of the surface lyses, cellular integrity could be maintained for a much longer time during which virus synthesis and release could continue.

It should be noted that the data here presented, while clearly establishing that the virus initiates a spreading disturbance on the red cell surface extending beyond the area covered by the virus particle itself, do not define the nature of the disturbance. It is possible that, as suggested by Burnet's (8) use of the term "browsing," each virus particle, itself, migrates over the active surface of the cell. However, this would require that as few as 9 virus particles will have moved over all of a cell's attachment surface and completed enzymatic digestion of each attachment site within a time less than 2 hours (Table IV B). Such an event seems difficult to conceive, in terms of random Brownian motion of the adsorbed virus particles. A scheme which appears more likely would involve triggering by the virus particle of a self-sustaining disturbance in the cell wall. Further experiments are required to clarify this step of the process.

The physicochemical basis of the requirement for a multiplicity of approximately 3 virus particles per red cell to effect receptor site destruction and at least the beginning of detectable elution within 24 hours is as yet unknown. Nor is it known whether this threshold requirement is absolute, for it is possible that a smaller number of virus particles could initiate this reaction in 48 or 96 hours. The extensive destruction which these cells undergo at 37°C. makes such measurements not feasible. It is conceivable that the existence of a threshold is an expression of the ability of the cell partially to reverse at least the early steps of the damage done by the virus. The lytic reaction set up by a single virus may be successfully compensated for by cellular action. When several viruses attack a single cell, the site-destroying action may overwhelm the cell's compensatory mechanism, and if so, the destructive reaction would proceed to completion.

The data which have been presented suggest a possible parallelism between the underlying reactions involved in the spontaneous elution of influenza virus from red cells and the phenomenon which has been called "lysis-from-without" in the bacteriophage system, a term used to describe a phage-induced destruction of the host-cell membrane which is unaccompanied by virus multiplication. In each case the effect takes place in a medium containing only inorganic salts; is inhibited by low temperature but not by ultraviolet inactivation of the virus; exhibits a threshold requirement of at least 3 viruses per cell; and evokes a disturbance causing loss of surface structures over an area much

greater than that covered by the virus particles which initiated the reaction (14, 15).

SUMMARY

Each chick and human red cell contains approximately 300 sites capable of attaching influenza virus particles. These correspond to an area representing approximately 2 per cent of the red cell surface.

Although the rate of attachment of PR8 to red cells is not diffusion-limited, when calculated on the basis of the total cell area, it does approach the theoretical maximum for interaction between the virus and the fraction of the cell area known to contain attachment sites.

It is demonstrated that the virus attachment can initiate a spreading disturbance on the red cell membrane which extends over an area far exceeding that covered by the attached virus and which leads to the destruction of receptor sites. This process does not involve cyclic virus attachment, elution from the cell, and reattachment to another site.

Practically all the receptor sites on a cell are destroyed before any virus is liberated into the medium.

The spontaneous elution of virus from red cells within 24 hours at 37° C. requires a threshold value of at least 3 and less than 5 virus particles per cell.

Parallelisms between the spontaneous elution reaction and the phenomenon of lysis-from-without in the bacteriophage system are demonstrated.

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