# THE NATURE OF THE VIRUS RECEPTORS OF RED CELLS

## II. The Effect of Partial Heat Inactivation of Influenza Virus on the Destruction of Red Cell Receptors and the Use of Inactivated Virus in the Measurement of Serum Inhibitor

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In a previous paper (1), it was shown that the influenza virus receptors of red cells and the virus agglutination inhibitor present in normal serum have many features in common. They are both destroyed by trypsin, sodium periodate in high dilution, and by influenza virus itself. They are both quite heat-stable and resist exposure to wide changes in hydrogen ion concentration. Of these similarities, the most striking and significant is the destruction by influenza virus. In the present paper, the adsorption and elution of influenza virus after heating at 56°C. will be described, together with the use of heated preparations for the measurement of serum inhibition. This method of measuring serum inhibition is such a great improvement over the use of unheated virus that the destruction of serum inhibitor by active virus can be much more conclusively demonstrated.

### *Methods*

The strains used in the present experiments were the PR8 strain of influenza A, the Lee strain of influenza B, and strain L230 which was isolated from the influenza A epidemic of 1947 and is characterized by the fact that it is inhibited by high dilutions of normal serum. Hemagglutinin titrations and serum inhibition tests were performed in the usual manner by the method of Hirst and Pickels (2). All the rabbit serum used was from a single pool and, unless otherwise indicated, was inactivated by heating at 56°C. for 30 minutes before use. All of the dilutions of serum were prepared in excess amounts so that they could be put into the final tubes without blowing out the pipettes. Pipettes were changed with each dilution. All titers are expressed as the reciprocal of the dilution and have been corrected for volume changes so that they can be compared directly. The hemagglutinating activity is occasionally referred to in terms of units. A unit may be defined as the amount of hemagglutinin which, in a final volume of 2 cc., will cause 50 per cent of the cells to agglutinate. The titer of a preparation (expressed as the reciprocal of dilution) is therefore the number of units contained in 2 cc. of the suspension.

### EXPERIMENTAL

It was observed by Francis (3) that Lee (B) virus, when heated at 56°C., lost little of its hemagglutinin titer but gave higher inhibition titers with human sera than did unheated virus in equivalent amounts. The effect was especially

noticeable in sera with low antibody levels. Although Francis was mainly concerned with the explanation of the phenomenon in terms of antibody measurement, it seemed very striking that a most marked effect was obtained with normal animal sera which contained no specific antibody. This suggested that the underlying mechanism of the differences in inhibition might lie in a change in the properties of the hemagglutinin itself, and to test this possibility the following experiments were performed.

TABLE I

*Effect of Heat on Adsorption and Elution of Influenza Virus from Red Cells*

| Strain | | Titer | Heated at 56°C. for | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0 min. | 5 min. | 10 min. | 15 min. | 30 min. | 60 min. |
| Lee(B) | 1 | Before adsorption | 104 | 74 | 64 | 69 | 64 | 69 |
| | 2 | Supernatant after adsorption | 13 | 6 | 6 | 6 | 5 | 5 |
| | 3 | Units of virus adsorbed | 91 | 68 | 58 | 63 | 59 | 64 |
| | 4 | Eluted virus | 84 | 49 | 37 | 24 | 2.5 | <2 |
| | 5 | Adsorbed virus eluted, *per cent.* | 92 | 72 | 64 | 38 | 4 | <3 |
| PR8(A) | 1 | Before adsorption | 169 | 138 | 104 | 112 | 84 | 56 |
| | 2 | Supernatant after adsorption | 37 | 52 | 42 | 37 | 20 | 16 |
| | 3 | Units of virus adsorbed | 132 | 86 | 62 | 75 | 64 | 40 |
| | 4 | Eluted virus | 104 | 45 | 39 | 37 | 30 | 14 |
| | 5 | Adsorbed virus eluted, *per cent.* | 79 | 52 | 63 | 49 | 47 | 35 |
| L230(A) | 1 | Before adsorption | 60 | 60 | 52 | 49 | 37 | 26 |
| | 2 | Supernatant after adsorption | 5 | 6 | 6.5 | 4 | 4 | 4.6 |
| | 3 | Units of virus adsorbed | 55 | 54 | 48.5 | 45 | 33 | 21.4 |
| | 4 | Eluted virus | 45 | 42 | 42 | 42 | 24 | <2 |
| | 5 | Adsorbed virus eluted, *per cent.* | 82 | 78 | 86 | 93 | 73 | <9 |

### *Effect of Heating on the Spontaneous Elution of Influenza Virus from Red Cells*

Influenza virus of three strains (Lee (B), PR8 (A), and L230) was heated at 56°C. for intervals up to 1 hour and the samples were tested for hemagglutinin titer (Table I, line 1). In each case, the heating caused a progressive decrease in titer. To each sample an equal volume of 1.5 per cent red cells was added at 0°C. and the mixture left for 30 minutes at this temperature. The cells were removed by centrifugation and the residual unabsorbed virus of the supernatant was tested (Table I, line 2). The centrifuged cells were then suspended in a volume of saline equal to that of the original virus sample and incubated for 3 hours at 37°C. The cells were again removed and the eluted virus in the supernatant titered.

The results with the Lee virus were the most striking. Heating this strain at 56°C. for 1 hour resulted in a 30 per cent drop in titer. The hemagglutinin in the various specimens was almost equally well adsorbed on red cells. Ninety-four per cent of the unheated virus eluted (Table I, line 3), while with

progressive heating the elution became less and less marked, and with virus heated for 1 hour, no detectable elution took place. There were similar changes with the other strains, although with the PR8 strain, the eluting ability was more heat-resistant.

TABLE II

*Effect of Heat on Adsorption and Elution of Influenza Virus from Red Cells*

| Strain | | Titered | Heated at 56°C. for | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0 min. | 5 min. | 10 min. | 20 min. | 30 min. | 40 min. | 60 min. |
| Lee(B) | 1 | Preliminary titration on concentrated virus..... | 1024 | 1175 | 1270 | 1560 | 1910 | 1910 | 2048 |
| | 2 | Before adsorption.. | 362 | 417 | 294 | 275 | 194 | 194 | 194 |
| | 3 | Supernatant after adsorption..... | 56 | 24 | 21 | 12 | 11 | 7 | 11 |
| | 4 | Units of virus adsorbed..... | 306 | 393 | 273 | 263 | 183 | 187 | 183 |
| | 5 | Eluted virus..... | 447 | 447 | 84 | 5 | <2 | <2 | <2 |
| | 6 | Adsorbed virus eluted, per cent. | 100* | 100* | 31 | 1.9 | <1.1 | <1.1 | <1.1 |
| PR8(A) | 1 | Preliminary titration on concentrated virus..... | 676 | 835 | 1024 | 1024 | 1175 | 1450 | 1450 |
| | 2 | Before adsorption.. | 779 | 722 | 512 | 479 | 362 | 338 | 447 |
| | 3 | Supernatant after adsorption..... | 45 | 24 | 13 | 10 | 10 | 5 | 7 |
| | 4 | Units of virus adsorbed..... | 734 | 698 | 499 | 469 | 352 | 333 | 440 |
| | 5 | Eluted virus..... | 1175 | 79 | 49 | 28 | 39 | 23 | 23 |
| | 6 | Adsorbed virus eluted, per cent.. | 100* | 11 | 10 | 6 | 11 | 7 | 5 |

* Significantly more hemagglutinin was eluted than was adsorbed.

In Table II, a similar experiment is recorded in which the virus suspensions used were concentrated by centrifugation and in which the titers of the concentrates increased on heating (line 1), possibly due to disaggregation. An attempt was made to use the same number of agglutinating units in each sample, based on these figures (Table II, line 2) but, during the lapse of time (20 hours) between the preliminary titrations and the final test, the hemagglutinin levels in the specimens heated longest dropped from their calculated level by an amount which would indicate that the specimens had returned to their original (preheated) level in that time. Nevertheless, the experiment shows clearly the same phenomenon revealed in Table I. Again, the PR8

strain showed greater resistance than the Lee strain in the loss of eluting capacity[1] but in this experiment, the decrease in elution could not be ascribed to a lack of sufficient adsorbed hemagglutinin.

These data (Tables I and II) and their interpretation form the basis for understanding the further experiments in this paper on the agglutinin inhibitor of normal serum. In terms of the author's enzyme-substrate hypothesis of the action of influenza virus on red cells (4), virus which has been heated at 56°C. still retains enough of its configuration to combine specifically with the substrate (adsorption on red cells) but has lost its enzymatic capacity to split it (lack of elution). Where influenza virus is active in splitting the receptor substance, the union between virus and cell is temporary and rapidly changing while with heated virus, where no receptor destruction takes place, the union is much more firm and binding. The consequences of this hypothesis are interesting in relation to experiments with serum inhibitor.

*Destruction of Serum Inhibitor by Influenza Virus as Measured by Heated (56°C.) Virus*

In a previous paper a number of experiments were described (1) which pointed to a strong basic similarity between the virus receptor of red cells and the normal inhibitor which occurs in many sera. The similarity included the destruction of inhibitor in the presence of active influenza virus. Since unheated virus destroys both serum inhibitor and red cell receptors the demonstration of inhibition in the presence of active virus is most complicated, because after mixing virus and serum the virus is probably not firmly bound to the inhibitor which it is constantly inactivating, and hence may become available for adsorption to red cells during the incubation period of the test. This labile combination of inhibitor and virus may lower the inhibition values obtained. If virus heated at 56°C. has lost the power of splitting the inhibitor as well as the red cell receptor the union between them should be firm, possibly irreversible, as shown in the previous experiments with red cells, and one would expect higher inhibitory levels with heated preparations, as Francis observed. The use of heated virus should offer more consistent values for the measurement of the serum-inhibiting effect.

*The Effect of Incubation Time on the Inhibition Obtained with Heated (56°C.) Virus.*—Before beginning tests on the destruction of virus inhibitor in serum, a

[1] The phenomenon of an increase in hemagglutinin titer immediately following heating, followed by a return to the former level after a period of hours has been repeatedly observed. In Table II it can also be seen that in some instances the titer of the eluted virus was higher than that of the adsorbed virus. These shifts in titer have been seen only with virus concentrated by centrifugation, in which case there is undoubtedly aggregation of virus particles. This is indicated by the frequent loss of a large part of the total hemagglutinin activity on centrifugal concentration. Apparently heating temporarily disperses the particles and elution does so more permanently.

preliminary experiment was done on the effect on the inhibitory titer of prolonged incubation of heat-inactivated (56°C.) virus with serum.

For this experiment the L230(A) strain was used which is notable for its high inhibition levels obtained with normal serum. A number of sets of serial dilutions of normal rabbit serum

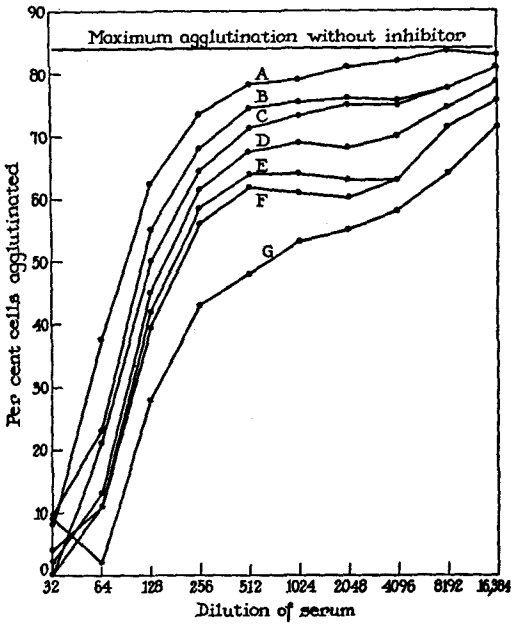


FIG. 1. Inhibition of hemagglutination by normal rabbit serum as tested with influenza virus, strain L230. The virus had been heated at 56°C. for 30 minutes and was used at a level of 12 units. Virus and serum dilutions were mixed and incubated at room temperature for various periods before the red cells were added. Line A, no incubation. Line B, 15 minutes incubation. Line C, 30 minutes. Line D, 1 hour. Line E, 2 hours. Line F, 3 hours. Line G, 20 hours incubation.

were made. Strain L230 (heat-inactivated 56°C.) was added to serum dilutions in a concentration such that the final hemagglutinin titer in each tube would be 12 units. The virus was added to sets of dilutions at appropriate intervals so that when cells were added to all the sets of dilutions the heated virus had incubated with the serum dilutions at room temperature for from zero minutes to 20 hours. Seventy-five minutes after adding the cells the tests were read. The degree of agglutination in each tube has been plotted in Fig. 1.

The results were striking and consistent throughout in showing that the virus which had been in contact with serum the least time before the addition of cells showed the least inhibition and that incubation of serum-virus mixtures resulted in progressively *increasing* inhibition up to the final 20 hour period. Whether equilibrium had been reached at 20 hours is not known. The increasing inhibition took place in all dilutions but was most marked at higher

dilutions. Virus control samples showed no significant drop in hemagglutinin titer when left at room temperature or in the ice box over the 20 hour period, and hence this factor probably does not enter into the explanation of the increase of inhibitory titer obtained. The increase in inhibition per unit time was most marked in the first 15 minutes and least in the final 17 hours, which is typical of adsorption curves in general.

This result obtained with heated virus is directly opposite to that previously obtained with incubation of active virus with serum (1), in that incubation with heated virus gave *increasing* inhibition while with unheated virus there was *decreasing* inhibition and this lends further support to the contention that with heated virus (56°C.) the ability to split the inhibitor is lost with retention of the capacity of the virus to combine with inhibitor.

The destruction of inhibitor in serum by active unheated virus could be further clarified if such treated sera could be tested with inactive virus. The problem in conducting such a test is to get rid of the unheated virus used for treatment so that it will not interfere with the heated virus of the final test. This may be done in two ways: (1) Treating serum with unheated virus and then heating the virus-serum mixtures after varying intervals at 56°C. in order to partially inactivate the virus and test the treated mixtures for inhibition with the virus heated in the presence of the serum. (2) Treating serum with unheated virus and heating after varying intervals at 65°C. in order to completely inactivate the virus present, and to conduct the inhibition tests with newly added virus which had been inactivated at 56°C. Both tests are useful and both have some disadvantages.

*Measurement of Inhibitor Destruction in Serum by Titration with Virus Inactivated in the Presence of Serum.*—

In this experiment a number of series of dilutions of normal rabbit serum were prepared. Active unheated Lee virus (18 units final concentration) was added at one time to a number of sets of dilutions, which after varying intervals were placed at 56°C. for 30 minutes. Before and after heating the mixtures were kept at room temperature; the longest period of incubation with unheated virus was 20 hours. Controls were tested in which previously heated virus (56°C.) was added to a set of serum dilutions 20.50 hours before cells were added (Fig. 2, line E) and in which preheated virus was added immediately before the addition of cells (Fig. 2, line D). Thus each set of dilutions, except line D, had had the same length of incubation at room temperature, the variables being the length of time before and after heating. Finally red cells were added to all the tubes and after 75 minutes the per cent of cells agglutinated was measured in every tube by a densitometer.

In such an experiment the question arises as to whether differences in inhibition might not, as in the previous experiment (Fig. 1), be due to differences in incubation time of serum with heated virus. Fortunately the Lee strain, used in this experiment does not show the large shifts in inhibition (lines D and E) with incubation which were found with strain L230. In addition, the

variation in incubation time in the experiments represented by lines B, C, and D was small (19.50, 20.25, and 20.50 hours respectively) and probably not significant in terms of equilibrium between heated virus and inhibitor. The drop in hemagglutinin titer of virus controls with heating at 56°C. was small

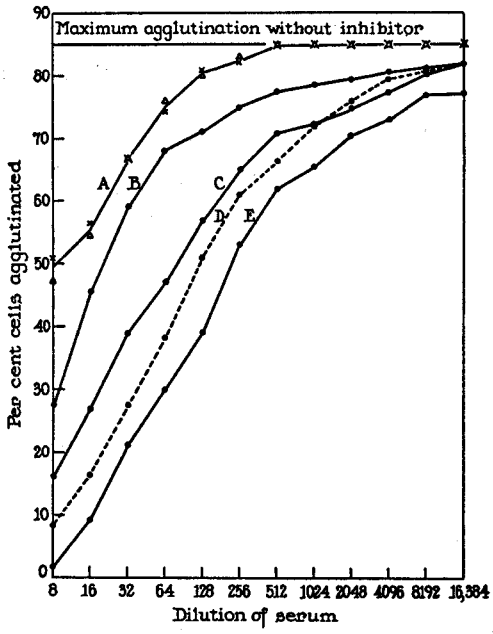


FIG. 2. Agglutination inhibition tests with normal rabbit serum, treated for various times with unheated Lee virus (18 units). The virus was added to the serum dilutions and inactivation was carried out at 56°C. for 30 minutes. The inhibition of the heat-inactivated virus was tested.

Line A (duplicate determinations), serum dilution treated for 20 hours with unheated virus and inactivated at 56°C. Line B, similar treatment in active state for 1 hour. Line C, treatment in active state for 15 minutes. Line D, preheated virus (56°C.) added to serum just before the test. Line E, preheated virus (56°C.) added to serum 20.5 hours before the test.

and uniform throughout the experiment. Though not indicated in the figure the inhibition effect was the same whether preheated virus was added to serum or the virus was heated immediately after addition to serum.

The exposure of normal rabbit serum to unheated virus resulted in a progressive and marked drop in the degree of inhibition obtained in a test with heat-inactivated virus. The decrease in inhibition was fairly uniform in all dilutions tested and whether lines D or E be taken as a base line for the inhibitory activity of untreated serum, the level dropped about 90 per cent in 3 hours and 95 per cent in 20 hours. While it is felt that this experiment fur-

nishes the most satisfactory evidence thus far for the inactivation of serum inhibitor by untreated virus, the method employed has the limitation that the shift in inhibition level must be measured by the same amount of the same strain which has been used for destroying the inhibitor. This objection is overcome in the second method.

*Measurement of Destruction of Serum Inhibitor by Active Virus Which Is Destroyed by Heating at 65°C. before Testing with Inactivated (56°C.) Virus.*— The advantages of destroying the active virus after it has acted on serum before

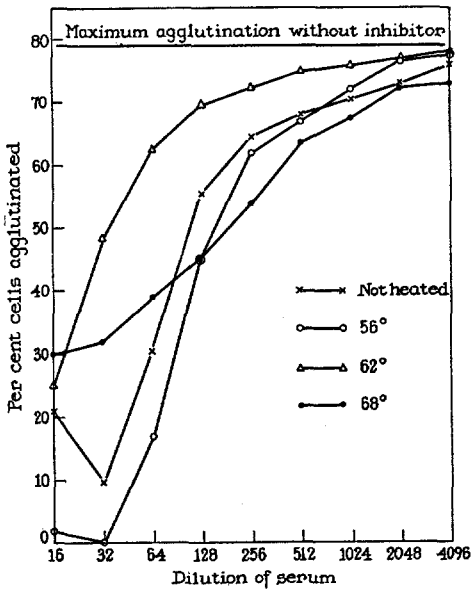


FIG. 3. Inhibition of agglutination of Lee virus by rabbit sera heated at several temperatures for 30 minutes. The sera were titered with 4.9 units of heated (56°C.) Lee virus.

testing with newly added virus are obvious since it multiplies the number of ways in which inactivated serum can be tested. This technique was not attractive at first because of the peculiar behavior of the inhibitor when serum is heated at temperatures above 60°C. McCrea has stated that the inhibitor of influenza virus hemagglutination is destroyed by heating for 15 to 20 minutes at 62°C. (5). We have not been able to confirm this statement and the behavior of inhibitor on heating is complex as will be shown below.

In a preliminary experiment, normal rabbit serum unheated, and heated at 56°, 62°, and 68°C. for 30 minutes was tested in inhibition tests with 5 units of heated (56°C.) Lee virus. The test (Fig. 3) shows no significant difference in inhibition curves between untreated serum and serum heated at 56°C. Serum heated at 62°C. showed a drop in inhibitory titer of about fourfold,

while serum heated at an even higher temperature (68°C.) showed a consistent and marked increase in inhibition over the level of the 62°C.) serum. At higher dilutions the serum heated at the highest temperature showed as much and possibly somewhat more inhibition than unheated serum but the shape of the curve is peculiar and the inhibition in lower dilutions is markedly less than that of normal serum. This is tentatively interpreted as interference with the inhibitor by various serum constituents, the interfering agents varying in activity with changes on heating, and their effect is diluted out more rapidly than the inhibition effect. Further evidence along this line will be offered in another paper.

In spite of the complicated behavior of inhibitor in serum heated at temperatures above 60°C. an attempt was made to use heat for inactivating virus in the presence of serum. Preliminary tests in the hemagglutinin titer variation with temperature showed a wide difference in susceptibility of various strains, and nothing less than 40 minutes at 65°C. would surely inactivate the hemagglutin of all preparations.

In this experiment two virus strains were used, Lee(B) and L230(A). Both were added in unheated state to normal rabbit serum. The final dilution of serum was 1:4 and the final titer of hemagglutinin with each strain was 32. These mixtures were allowed to stand at room temperature for 20 hours. Samples were removed periodically and heated at 65°C. for 40 minutes. The same procedure was carried out with serum and heat-inactivated preparations (56°C.). In addition, controls of serum (without virus added) heated at 65°C. and at 56°C. were tested. All specimens were titered for inhibition with 3 units of heat-inactivated virus (56°C.) of the two strains used. The results are shown in Figures 4, 5, 6, and 7.

In Fig. 4 the results of treating serum with heated or unheated Lee virus and testing with heated (56°C.) Lee virus are shown. Lines D and E show the effects of heating serum alone at 65°C., the titer being reduced about twofold over the 56°C. control. Curves A, B, and C show that the effect of incubating serum with unheated Lee virus was a marked reduction in the inhibitory level, similar to that seen in Fig. 2. Since these curves are not parallel, but converge in the higher serum dilutions, it is not clear how much inhibitor was destroyed. At the 60 per cent agglutination level the destruction would appear to be only 30 per cent in 1 hour while at the 10 per cent level it is close to 90 per cent. This convergence of curves cannot be explained satisfactorily at present but may be due to multiple factors affecting the inhibition as suggested above. When the same sera were tested with a second virus (L230) the convergence was not marked.

When virus (Lee) which had been heated at 56°C. was added to normal rabbit serum and inactivated at 65°C. after varying intervals up to 20 hours there was no change in inhibitory level and the curve at 20 hours (Fig. 4, line D, triangles) coincided with that of untreated serum heated at 65°C. (line D, ×), thus confirming the fact that partially heat-inactivated virus (56°C.) has lost

its capacity to destroy inhibitor. A further control of active virus added to serum and inactivated at 65°C. at once gave values coincident with line D.

Fig. 5 shows the results of testing the same serum samples of the previous experiment (lines A, B, C, and D in Fig. 4) with another virus, strain L230A). The inhibition curves with this strain have a characteristic appearance with a

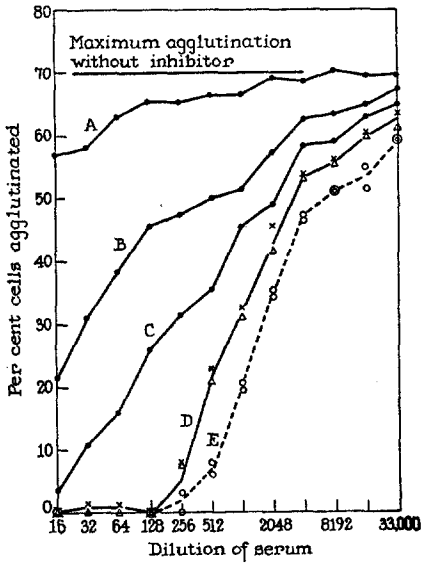


FIG. 4. Agglutination inhibition tests with normal rabbit serum treated for various periods with unheated Lee virus. The virus was destroyed by heating the serum-virus mixtures at 65°C. for 40 minutes and the sera were tested for inhibitory titer against 3 units of heated (56°C.) Lee virus. This amount of Lee virus was sufficient to agglutinate 70 per cent of the red cells in the absence of any inhibitor.

Line A, serum treated with unheated Lee virus for 20 hours. Line B, treated for 3 hours. Line C, for 1 hour. Line D (×), for zero hours. Line D (triangles), serum treated with heated (56°C.) Lee virus for 20 hours. Line E (open circles, duplicate determinations), control serum, no virus added and heating to 56°C. only.

plateau in the center and as used with these sera are consistent with the maximum inhibitor destruction found with the homologous (Lee) strain. The curves are much more nearly parallel than with the Lee strain and show 60 to 75 per cent destruction of inhibitor in 1 hour, over 90 per cent in 3 hours, and more than 99 per cent in 20 hours

In Fig. 6 are shown the results of treating the same normal rabbit serum with strain L230 at the same strength in terms of agglutinating units as the Lee strain in the experiment above. This is a newly isolated strain which shows very high inhibition levels with normal serum in the unheated state, much

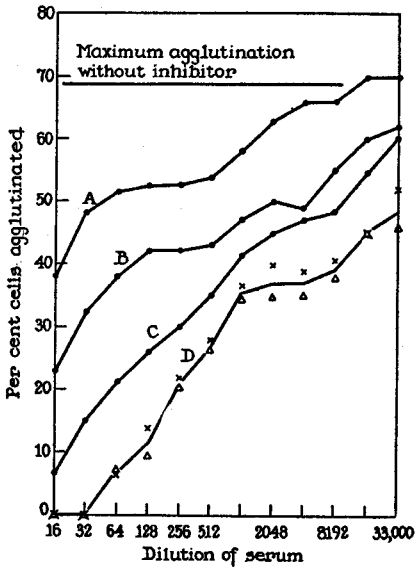


FIG. 5. The sera are the same ones tested in Fig. 4. In this experiment they were tested for inhibitory titer with strain L230 heat-inactivated at 56°C. and used at 3 units.

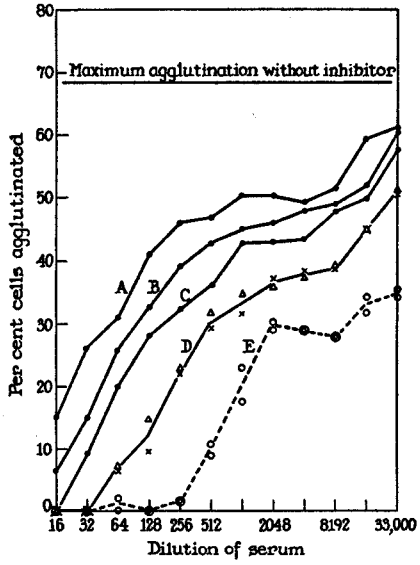


FIG. 6. Agglutination inhibition tests with normal rabbit serum treated for various periods with unheated L230(A) influenza virus. The virus was destroyed by heating at 65°C. for 30 minutes and the sera were tested for inhibitory titer against strain L230 heated (56°C.).

Line A, exposure of serum to unheated virus for 20 hours. Line B, for 3 hours. Line C, for 1 hour. Line D (×), for zero hours. Line D (triangles), exposure to heated (56°C.) L230 virus for 20 hours. Line E (open circles, duplicate determinations) control serum, no virus added and heating to 56°C. only.

higher than Lee or PR8. One might deduce from this that this strain splits the inhibitor at a lower rate than the Lee strain. However, its elution from red cells is almost as rapid as that of the Lee strain and the difference in rate of destruction shown in Fig. 6 is only slightly less than the rate with Lee, almost 75 per cent in 1 hour and over 90 per cent in 20 hours. Again, as in Fig. 5, the curves have a characteristic plateau; within the limits of experimental error they are parallel and serum treatment with heated L230 virus (56°C.) had no effect on the inhibitory titer. Heating normal untreated serum at 65°C.

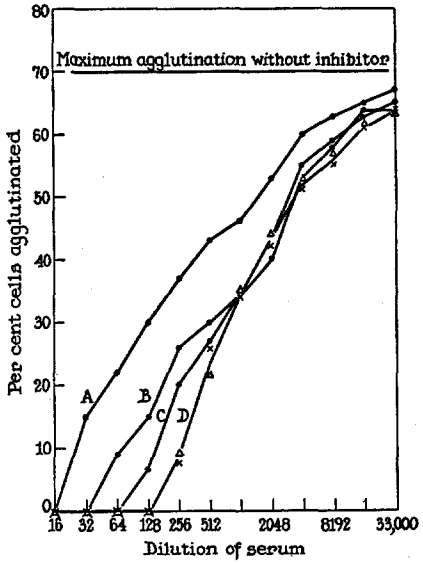


FIG. 7. The sera are the same as those tested in Fig. 6. In this experiment the sera were exposed to unheated virus of strain L230 but were tested for inhibitory titer with heated (56°C.) strain Lee, 3 units.

lowered the inhibitory titer by 75 per cent for this strain as opposed to 50 per cent for the Lee strain which is a significant difference. The same L230-treated sera when tested with the Lee strain gave the picture seen in Fig. 7. In the region of 10 to 20 per cent agglutination the reduction of inhibitor was consistent with the amount of inhibitor destruction seen in Fig. 6, but the curves converged very markedly in the zones of lesser inhibition. Again it seems likely that this lack of parallelism may be due to interfering serum factors, or possibly, multiple inhibiting factors, one of which affects the Lee strain at high dilution more than it affects agglutination by the L230 strain.

## DISCUSSION

The removal of the destructive or enzymatic capacity of a virus without completely destroying its capacity to combine with red cell receptors or serum

inhibitor provides a unique tool for the quantitation of the inhibitory action of serum on virus hemagglutination. By means of inhibition titrations with active virus it has been possible to demonstrate more conclusively than before that unheated virus has the ability to rapidly destroy the virus inhibitor in serum. While it may be questioned at this time whether it is the virus or something else in virus suspensions which destroys the serum inhibitor the bulk of evidence at the moment is in favor of the association of the activity with the virus particle. Virus partially purified (by centrifugation or by adsorption on an elution from red cells) also possesses the inhibitor-destroying capacity, while very low titer supernatant fluids from which the virus has been removed by centrifugation do not. A more precise proof that the activity is associated with the virus itself may be possible after purification of the serum inhibitor.

The use of heat-inactivated influenza virus for measuring serum inhibitor provides a rather accurate means of titrating this factor and one which is also very sensitive. In tests in which the L230 strain was used and left in contact with the serum dilutions for several hours a significant degree of inhibition of agglutination has been detected in sera diluted over 100,000 times. Assuming that rabbit serum contains a 1 per cent concentration of this substance, the limit of detection would be of the order of part 1 in 10 million.

The use of inhibition curves in this paper might have been avoided by the use of simple end points (*e.g.* the dilution of serum at which the agglutination of red cells is 50 per cent complete), in which case the results obtained would have been qualitatively similar to the ones presented. However, the entire curves tell a more complete story and this is especially important where the inhibitor is being measured in whole serum and heated serum, because of the complicating factors which interfere with the inhibition.

Of the two methods of measuring inhibitor destruction by unheated virus the second is more versatile. When testing for inhibitor in whole serum the marked degree of loss in inhibitor on heating at 65°C. is disturbing. As will be shown in another paper, this objection does not enter into the titration of purified inhibitor.

Burnet and his colleagues (6, 7) have published two preliminary notes in which they refer to a method of measuring serum inhibitor with heat-inactivated virus but at the time of the present report a detailed description of their method was not available to the author.

## SUMMARY

Evidence has been offered that influenza virus which has been heated at 56°C. for 30 or more minutes loses some of its capacity to agglutinate red cells and may completely lose its power to elute from cells on which it has been adsorbed. Such heat-inactivated virus does not possess the capacity to

destroy the virus inhibitor in normal rabbit serum and this appears to be the explanation of the higher agglutinin inhibitory levels obtained with serum and heated virus as compared with serum and untreated virus.

The heat-inactivated virus can be used to measure the inhibitor substance in normal rabbit serum. By two different methods it has been demonstrated that the inhibitor is destroyed in the presence of unheated influenza virus, as measured by inhibition titrations with virus inactivated at 56°C. The destruction of inhibitor by virus of either type A or B can be measured by virus of either type with similar results.

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