# QUANTITATIVE ASPECTS OF THE RED BLOOD CELL AGGLUTINATION TEST FOR INFLUENZA VIRUS*

By GAIL LORENZ MILLER, Ph.D., and W. M. STANLEY, Ph.D.

*(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)*

(Received for publication, November 3, 1943)

During the isolation and purification of biologically active materials it is of the utmost importance that a means for measuring specific biological activity be available, since such measurements can be used as an index of chemical purity and hence as a guide in connection with concentration and purification procedures. In order that such tests may be significant, it is essential that the substances under study do not lose activity during the period required for the isolation and the test, and that the biological test be sufficiently sensitive and accurate. In the case of influenza virus, the fact that virus preparations can lose activity rapidly (1) and especially the fact that tests of infectivity are accurate only to within several hundred per cent (2) preclude the use of infectivity tests as a significant criterion of chemical purity of the virus. However, the discovery of Hirst that chicken red blood cells are agglutinated by the allantoic fluid of chick embryos infected with influenza virus (3), together with evidence that the agglutination factor may be identical with the infectious unit of the virus (3–6), presented the possibility of using the method for the quantitative estimation of virus. It must be noted that under certain conditions of heat or chemical treatment (5) or of hydrogen ion concentration or simply standing with time (7), the infectivity of influenza virus may be lost whereas the agglutination activity may remain essentially constant. It is therefore apparent that the agglutination test cannot be regarded as a direct measure of active virus. It should also be noted that, in contrast to the mouse (2) or chick embryo (8) methods of testing for virus activity, a relatively concentrated solution of virus is required for tests by the red cell agglutination method. However, because of the rapidity and ease with which the agglutination test can be conducted, it appeared to offer special advantages in research on influenza virus, especially when such research is carried out rapidly under conditions of maximum virus stability and when it can be shown by other methods that the virus preparations have a high infectivity. Accordingly, a detailed study was made of the nature of the variables inherent in the method in order to determine whether a procedure of measurement might be established

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research.

by means of which results of reproducibility sufficient to be employed as an index of chemical purity of virus preparations could be obtained.

In Hirst's method a solution containing hemagglutinin is diluted to a point where it causes one-half of the red cells in a 0.75 per cent suspension to agglutinate and settle out, under specific conditions which are described, in 75 minutes. This amount of hemagglutinin is defined as one unit, and the titer of a given fluid is then expressed as the number of times the original fluid must be diluted in order to reduce the concentration of hemagglutinin to one unit. In work with purified protein preparations containing virus activity, we have found it convenient to measure units of chicken red cell agglutination (CCA) activity in terms of unit weight of protein nitrogen. Thus, the number of units of CCA activity per milligram of protein nitrogen in a given preparation is the volume to which a solution containing 1 mg. of protein nitrogen per cc. must be diluted in order that 1 cc. will yield the prescribed end point under standard conditions.

Hirst and Pickels have shown that a number of factors must be carefully controlled in the quantitative measurement of agglutination titer if significant results are to be expected (9). For example, it is of importance to make readings of densities of mixtures of virus and red cell suspensions at the correct height in the tubes used in the test and also to carry out the measurements after an exact period of incubation. We have confirmed the importance of these precautions. Hirst and Pickels reported that duplicate determinations usually fell within a very narrow range, although the variation was somewhat greater when determinations on the same virus samples were carried out on different days with different stock suspensions of red cells. We have encountered this same sort of variation and have therefore made special studies in order to ascertain the causes of the variation. In the present report are described the results of studies on (*a*) the accuracy in replicate measurements, (*b*) the effect of pH, (*c*) of temperature, (*d*) of concentration of red cells, (*e*) of red cells from different chickens, and (*f*) of red cells of different ages on the reaction, and (*g*) the stability of the CCA activity of influenza virus preparations. On the basis of the findings of these studies, a method of establishing a reproducible standard of CCA activity and a standard procedure for carrying out the test are presented.

## *Materials and Methods*

*Agglutination Titrations.*—Hirst and Pickels (9) have described a special densitometer which was constructed for the measurement of agglutination titers. In the present work it was found that, with the aid of a small wooden adapter fitted with a spring clip to hold the agglutination tubes securely, it was possible to utilize a standard, commercially available Klett-Summerson photoelectric colorimeter for the purpose of the density measurements. A green filter No. 54 was used in the colorimeter. When the agglutination tubes were adjusted to the correct height in the beam of

light, a 0.37 per cent suspension of red blood cells gave a colorimeter reading of about 350 and in general the results which were obtained were entirely comparable to those obtained by Hirst and Pickels. The test tubes employed were set up in a wooden block which held the tubes in an exactly vertical position. This was of importance since it prevented the agglutinated cells from sticking to the walls of the test tubes.

For making serial dilutions, advantage was taken of the up-and-down movement of the plunger in the syringe of an automatic pipetting machine, and by means of a special pipette connected to the syringe an automatic mixing apparatus was devised. Uniform serial dilutions were more readily obtained with this apparatus than by the usual methods. The titrations were in other respects carried out according to the directions of Hirst and Pickels.

*Chicken Red Cells.*—The red cells employed in the present study were obtained from adult chickens, usually by severing the cervical vessels, and were prepared according to the directions of Hirst and Pickels (9). The samples were stored at 4°C. in the form of a 15 to 30 per cent suspension in saline.

*Source of Virus.*—Samples of purified PR8 influenza virus were prepared by differential centrifugation (10). Before being used in the titration studies, the final preparations were diluted with 0.85 per cent sodium chloride to a point where subsequent dilution to the range of 1:4 to 1:16 yielded the end point of the titration. Although 0.1 M phosphate buffer at pH 7 has been found to be preferable to saline as a diluent in tests of influenza virus activity using mice and chick embryos (11), no great advantage was achieved by the use of the buffer in the agglutination tests. This fact will be shown later in the Experimental section of this paper.

## EXPERIMENTAL

### *The Accuracy in Replicate Measurements*

Hirst and Pickels (9) reported that in a large number of CCA measurements carried out in duplicate, 90 per cent of the pairs showed differences in end points of not more than 6 per cent and few gave differences greater than 12 per cent. In order to establish more precisely the significance of differences in end points, we have carried out the following series of titrations and have subjected the results to statistical analysis. The first experiment consisted of three titrations, each composed of 5 replicas and differing in concentrations of virus by the proportions, 120, 100, and 80, respectively. In a second experiment carried out at a later date with a different sample of virus and of red cells, the amounts of virus in the titrations were adjusted to the proportions of 100, 90, and 80, respectively. End points were calculated for the individual replicas and mean end points were calculated for each titration. The data are presented in Table I, from which it may be estimated that the values of the mean end points obtained in Experiment 1 were in the proportions of 121, 100, and 82, respectively, and for Experiment 2, the corresponding values were in the proportions of 100, 88, and 82, respectively.

When account is taken of the differences in concentrations of virus in the titrations within a given experiment and the differences between the end points of the two experiments, a standard error of the mean of duplicate analyses of

TABLE I

*Accuracy in Replicate Tests of Agglutination Titers of Preparations Differing by 10 and 20 Per Cent in Virus Concentration*

| Experiment No. | Proportions of virus in test samples | Colorimeter readings | | | | | End points of individual tests | Mean end points |
|---|---|---|---|---|---|---|---|---|
| | | 50 per cent standard | Dilution of test samples | | | | | |
| | | | 1:4 | 1:8 | 1:16 | 1:32 | | |
| | | | | | 230 | 380 | 27.8 | |
| | | | | | 232 | 375 | 28.6 | |
| | 120 | 355 | | | 240 | 390 | 26.8 | 28.6 |
| | | | | | 220 | 365 | 30.0 | |
| | | | | | 232 | 365 | 29.8 | |
| | | | | | 258 | 400 | 25.2 | |
| | | | | | 264 | 450 | 22.2 | |
| 1 | 100 | 355 | | | 264 | 410 | 24.2 | 23.6 |
| | | | | | 262 | 410 | 24.2 | |
| | | | | | 268 | 435 | 22.4 | |
| | | | | | 300 | 420 | 20.4 | |
| | | | | | 308 | 465 | 19.4 | |
| | 80 | 355 | | | 306 | 458 | 19.6 | 19.4 |
| | | | | | 330 | 455 | 18.0 | |
| | | | | | 308 | 445 | 20.0 | |
| | | | 264 | 435 | | | 5.56 | |
| | | | 238 | 415 | | | 6.03 | |
| | 100 | 345 | 264 | 430 | | | 5.56 | 5.79 |
| | | | 252 | 415 | | | 5.89 | |
| | | | 256 | 415 | | | 5.89 | |
| | | | 274 | 470 | | | 5.13 | |
| | | | 274 | 465 | | | 5.19 | |
| 2 | 90 | 345 | 274 | 470 | | | 5.13 | 5.10 |
| | | | 278 | 465 | | | 5.13 | |
| | | | 276 | 485 | | | 4.90 | |
| | | | 305 | 495 | | | 4.63 | |
| | | | 310 | 490 | | | 4.57 | |
| | 80 | 345 | 300 | 480 | | | 4.74 | 4.73 |
| | | | 284 | 475 | | | 4.95 | |
| | | | 305 | 465 | | | 4.74 | |

2.9 per cent can be estimated from the data as a whole. From this value of the standard error it can be shown that, in any particular case where duplicate analyses are made upon different samples, the chances are 19 out of 20 that differences of 8.4 per cent in the mean end points are significant.

The high degree of accuracy attainable under the conditions described is apparent from the data above mentioned. The accuracy indicated is valid, however, only with respect to the simultaneous comparison of different samples. As will be shown later, the absolute numerical value of the titer of a given sample of virus may vary considerably under different sets of conditions.

### *The Effect of pH on the Reaction*

In order to test the effect of pH on the CCA reaction, 0.1 M phosphate buffers at pH 6–9 were prepared and used for making serial twofold dilutions of a sample of influenza virus. Freshly prepared red cells were made up in 0.85 per cent sodium chloride in the usual way. Five replicate titrations were carried out at each pH and the mean end points were calculated from the data obtained. The final values of the end points for the tests at pH 6, 7, 8, and 9 were in the proportions of 105, 100, 100, and 91, respectively. The experiment was repeated and the corresponding end points were this time found to be in the proportions of 102, 100, 97, and 83. It is apparent from these results that the pH of the medium has only a minor effect over the range of pH 6–8 but that significantly lower titers may be obtained at pH 9. Errors of measurement arising from the use of unbuffered saline as a diluent for CCA tests are therefore quite unlikely, particularly if the original sample of virus being tested is near pH 7 in a buffered medium. In control experiments in which the results obtained with the use of phosphate buffer at pH 7 as a diluent were compared directly with those obtained with saline, it was found that titers approximately 18 per cent lower were obtained in the former case. It was concluded that the use of the phosphate buffer as a diluent offered no great advantage for the CCA tests.

### *The Effect of Temperature on the Reaction*

Hirst mentioned that the agglutination tests were carried out at room temperature (5) but apparently made no detailed study of the effect of temperature on the titration results. Since room temperatures may vary by as much as 10° or more, it appeared desirable to determine the effect of temperature on the test. In the present studies, in which agglutination measurements were made in replicate at temperatures ranging from 4° to 35°C., it was found that the most reproducible values were obtainable at about 24°C. For simplification, the values obtained at this temperature were given an arbitrary value of 100, and the data obtained at other temperatures were recalculated on this basis. The results are summarized in Table II, from which it is apparent that the temperature exerts a direct influence on the values of the titers. Furthermore, the effect was common to red cell preparations from different sources and of different ages.

The data shown in Table II can be plotted graphically and a smooth curve fitted to the points on the graph. The best straight line which can be drawn on the curve between the temperatures of 20° and 30° indicates that the value of the titer changes about 4.5 per cent per degree above and below 25°C. Because of the magnitude of the effect, it is proposed that a standard tempera-

ture of 25°C. be established for measurements of CCA activity. The formula for the calculation of the corrected CCA titer on this basis is therefore

$$CCA_{25°} = \frac{100}{100 + (t_1 - 25)(4.5)} \times CCA_{t_1}$$

where $CCA_{25°}$ represents the corrected CCA titer, $t_1$ represents the temperature of the measurement, and $CCA_{t_1}$ represents the experimentally observed CCA titer.

### *The Effect of Concentration of Red Cells*

A concentration of red cells of 1.5 per cent was chosen by Hirst and Pickels (9) for the agglutination test because of the fact that the densitometer used in the ag-

TABLE II

*Effect of Temperature on the CCA Test*

| Experiment No. | Preparation of virus | Preparation of blood cells | Age of blood cells | Relative CCA titers at different temperatures | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 4°C. | 16°C. | 20°C. | 24°C. | 30°C. | 35°C. |
| | | | *days* | | | | | | |
| 1 | 11I | A | 1 | | | 85 | 100 | | 180 |
| 2 | 11I | A | 2 | | | 81 | 100 | 120 | |
| 3 | B9 | C | 1 | 41 | 77 | | 100 | | 145 |
| 4 | B9 | D | 2 | 38 | 50 | | 100 | | 165 |
| 5 | B9 | D | 6 | 21 | 51 | | 100 | | 162 |
| 6 | B9 | F | 1 | 30 | 57 | | 100 | | 197 |
| 7 | 10I | I | 2 | | | 80 | 100 | 120 | |
| 8 | 10I | J | 7 | | | 83 | 100 | 116 | |
| 9 | 10I | K | 3 | | | 75 | 100 | 131 | |
| Averages | | | | 33 | 59 | 81 | 100 | 122 | 170 |

glutination measurements appeared to be most sensitive under this condition. The use of a 1.5 per cent concentration of red blood cells has also proved very satisfactory for determination of CCA titers with the Klett-Summerson colorimeter which we have employed. It was of interest, however, to ascertain whether it was essential to the standardization of the agglutination method to employ exactly a 1.5 per cent suspension of red cells. In a preliminary experiment, suspensions of red cells of concentrations of 1.29, 1.37, 1.46, 1.54, 1.63, and 1.71 per cent were prepared. The 50 per cent end points yielded by aliquots of a stock sample of virus were then measured with each suspension. The results from each measurement were calculated on the basis of the 50 per cent standard reading given by the corresponding red cell suspension used in the measurement, and the end points were found to be 3.24, 3.76, 3.72, 3.39, 3.24, 3.31, respectively. It appeared therefore that changes in red cell concentration over a limited range are not accompanied by significant changes in end points.

The experiment was repeated with a greater range of concentrations of red blood

cells, namely, 0.43, 0.64, 0.87, 1.07, 1.29, 1.50, 1.71, 1.93, 2.14, 2.57, and 3.00 per cent and the corresponding titers which were obtained were 2.12, 4.07, 3.75, 3.84, 3.47, 3.70, 4.24, 3.36, 3.15, 3.09, and 3.52. With the exception of the lowest concentration of cells used, the results again indicated no significant tendency for the end points to vary with changes in concentration of red cells. It may be concluded, therefore, that the small variations in concentration of red cells which may be encountered in the preparation of cells for routine measurements of CCA activities will introduce no error into the titrations, provided, of course, the final results are calculated on the basis of the 50 per cent reading of the particular suspension of cells employed for the test.

TABLE III

*Effect of Red Cells from Different Chickens on the CCA Test*

| Experiment No. | Preparation of red cells | Relative CCA titers |
|---|---|---|
| 1 | G (hen) | 78 |
| | H " | 86 |
| | I " | 99 |
| | J " | 110 |
| | K " | 112 |
| | L " | 114 |
| 2 | M (rooster) | 82 |
| | N (hen) | 90 |
| | O (rooster) | 91 |
| | P " | 103 |
| | Q " | 105 |
| | R " | 111 |
| | S " | 120 |

It is of theoretical interest that these studies demonstrate that a given amount of virus will cause the agglutination and settling out of a constant percentage of red cells per unit time over a very wide range of initial concentration of the red cells.

### *The Effect of Red Cells from Different Chickens*

Samples of red blood cells, obtained from six different adult hens, were prepared. After one day of standing at 4°C., each sample of cells was used in agglutination tests which were carried out on aliquots of a suitable dilution of influenza virus. For ease of comparison, the average of the titers obtained with the various blood samples was given an arbitrary value of 100 and each value was then recalculated on this basis. The results of this experiment and of a duplicate experiment with blood cells from six roosters and one hen are presented in Table III. The maximum variations in titers were 36 and 38 per cent in Experiments 1 and 2, respectively. This variation is too great to permit the use of different samples of red cells in quantitative measurements that are used to evaluate the chemical purity of an influenza virus preparation.

This and other considerations to be discussed later indicate that a preparation of virus rather than a preparation of red cells should be used for purposes of standardization.

### *The Effect of Red Cells of Different Ages*

In preliminary tests, fresh samples of red cells were prepared at weekly intervals and were tested from time to time for ability to be agglutinated by a given preparation

TABLE IV

*The Effect of Red Cells of Different Ages on the CCA Test*

| Experiment No. | Preparation of blood cells | Relative CCA titers obtained with red cells of different ages Days | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 17 |
| 1 | A | 100 | | 72 | | | | | | | | |
| | B | 100 | | 89 | | | | | | 16 | | |
| | C | | 100 | | | | 106 | 82 | | | | |
| | D | | 100 | | | | 109 | 77 | | 55 | | |
| | E | | 100 | | | | | 71 | 41 | | | |
| | F | | 100 | | | 98 | | | | | 106 | |
| 2 | G | | 100 | 88 | 81 | 85 | | | 52 | | | |
| | H | | 100 | 98 | 76 | 77 | | | 49 | | | |
| | I | | 100 | 90 | 74 | 67 | | | 43 | | | |
| | J | | 100 | 83 | 78 | 67 | | | 51 | | | |
| | K | | 100 | 96 | 70 | 66 | | | 62 | | | |
| | L | | 100 | 84 | 67 | 70 | | | 47 | | | |
| 3 | B | 100 | | | | | | | | 16 | | |
| | B (packed) | | | | | | | | | 42 | | |
| | C | | 100 | | | | | 82 | | | | |
| | C (packed) | | | | | | | 93 | | | | |
| | E | | 100 | | | | | | 41 | | | |
| | E (packed) | | | | | | | | 33 | | | |
| | F | | 100 | | | | | | | | 106 | |
| | F (packed) | | | | | | | | | | 106 | 53 |

of purified influenza virus. The titers obtained in the first measurements which were carried out with each preparation of red cells were given an arbitrary value of 100, and values obtained in subsequent titrations were recalculated on this basis. All titers were corrected for temperature, as described in a previous section. The results are summarized under Experiment 1 of Table IV. Preparations of red cells A and B showed slight deterioration in 2 days, with marked loss in ability to agglutinate in the latter case at the end of 8 days. Preparations C, D, and E appeared to be stable for about 5 days but then deteriorated. Preparation F yielded constant titers for 9 days. A second series of experiments was next carried out, in which samples of blood cells from six different chickens were prepared at the same time under

the same conditions and were then tested at intervals for ability to be agglutinated by a given sample of influenza virus. As shown by the data for Experiment 2 in Table IV, on the average about 50 per cent loss in ability to agglutinate occurred during 7 days. The figure of approximately 50 per cent loss in 7 days has been confirmed repeatedly in miscellaneous titrations not shown in Table IV.

Hirst, in preparing red cells, has stored the washed cells in a packed condition (4). In order to test whether the cells might deteriorate less rapidly under such conditions, control samples of blood in certain of the tests shown in Experiment 1 of Table IV were packed in a centrifuge tube and were set aside for a period of time before they were retested. The results of these tests, presented under Experiment 3 of Table IV, indicate that storing the red cells in a packed condition did not contribute greatly to the stability. It was concluded from the data as a whole that red cells cannot be depended upon to yield reproducible results during storage after preparation.

### *The Stability of the CCA Activity of Influenza Virus*

Tests for stability of the CCA activity of influenza virus when stored at 4°C. were carried out on 11 different preparations of virus purified by differential centrifugation. The results of the preliminary series of tests are presented as Experiment 1 in Table V. For ease of comparison, the average titer obtained for each sample was given an arbitrary value of 100 and the titers found were recalculated on this basis. The titers shown in Experiment 1 were not corrected for temperature and special attention was not paid to the use of freshly prepared red cells, since the importance of these factors was not realized at the time the preliminary tests were carried out. When account is taken of variation which may be attributed to these factors, the data reveal no significant change in CCA titer of any of the preparations during the periods of time over which the tests were conducted, namely, 8 to 53 days. The results represented by Experiment 2 in Table V were corrected for temperature and were obtained with preparations of red cells not over one day old. The data of this experiment provide confirmation for the conclusion derived from the data of Experiment 1 and reveal further that preparations of virus may maintain constant CCA activity for as long as 8 months at 4°C.

It appeared that a maximum stability of the CCA activity of influenza virus might be insured if samples of the virus were frozen and stored at −70°C. Preliminary studies have indicated, however, that the CCA activity of the virus is less stable under these conditions than when stored at 4°C. For example, the B9 virus preparation, frozen in a 0.1 per cent solution in 0.1 M phosphate buffer, showed a constant CCA activity when thawed and tested at intervals during the first month of storage, but thereafter on comparison with samples stored at 4° revealed in repeated tests a 30 to 40 per cent decrease in titer. Similar results have also been obtained with other preparations of virus. It was concluded, therefore, that storage of the influenza virus at 4° provided the more favorable condition for the stabilization of the CCA activity.

### *Establishment of the Standard of CCA Activity*

The studies described in the above sections have demonstrated that the variability in results encountered in agglutination tests can be attributed to the variation in the behavior of red cells from different chickens and to the instability of the red cells themselves. Preparations of purified influenza virus, on the other hand, were demonstrated to be stable for several months with respect to CCA activity and apparently yielded variable results only because of the variability of the red cells used in the tests. This fact, together with the accuracy of measurement which was found to be obtainable in replicate titrations, made it possible to establish a reproducible standard unit of CCA activity.

TABLE V

*Stability of the CCA Activity of Influenza Virus on Standing at 4°C.*

| Experiment No. | Preparation of virus | Age of virus at start of experiment | Relative CCA titers at times after start of experiment Days | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1–4 | 5–9 | 10–15 | 16–22 | 23–30 | 31–43 | 53–55 | 162 |
| | | *days* | | | | | | | | | |
| | A8 | 0 | 85 | 85 | 130 | | | | | | |
| | B9 | 0 | | 88 | 126 | | | | 71 | 115 | |
| | C6 | 0 | | 101 | 99 | | | | | | |
| | C7 | 0 | | 126 | 84 | | | | 109 | 81 | |
| 1 | D7 | 0 | | 94 | 106 | | | | | | |
| | F8 | 0 | | 121 | 113 | 102 | | 71 | 96 | | |
| | G10 | 0 | | 103 | 89 | 61 | | 124 | 123 | | |
| | H10 | 0 | | 105 | 105 | 105 | | 83 | 102 | | |
| | I10 | 0 | | 89 | 114 | 101 | | 97 | | | |
| | B9 | 83 | 109 | | 86 | 111 | | | | 96 | 98 |
| 2 | 11I | 5 | 109 | | 101 | 87 | 109 | 81 | 112 | | |
| | M6 | >50 | 109 | | 90 | 84 | 101 | 95 | 122 | | |

The B9 preparation of influenza virus, described earlier, was chosen for a standard of CCA activity. The average titer, calculated from the results of tests carried out with ten different preparations of freshly washed red cells, was 26,000 units per mg. of protein nitrogen corrected to the standard temperature of 25°C.

The standardized procedure for carrying out quantitative measurements of CCA activity may be summarized as follows: A sample of purified influenza virus, standardized as described above, is employed as the standard for the measurements and is titered simultaneously with titrations of unknown samples. Since the activity of the standard virus preparation is corrected for temperature, no further temperature correction is necessary and the CCA activity of an unknown sample is obtained directly by multiplying the value of the apparent titer for the unknown by the ratio of the theoretical titer of the standard to the apparent titer of the standard. The

effects of variations due to different sources and different ages of red cells and to different temperatures of measurement are thus eliminated automatically. From the standpoint of the estimation of the probable end point of a given sample of virus before a dilution series is set up, it is most practicable to employ red cells not over one week old, since under these conditions the stability of red cells is usually sufficient that the apparent titers obtained will not be altered more than twofold. Furthermore, when a dilution series is set up, the solution of virus being tested is first diluted directly with saline to a point where subsequent dilution to the range of 1:4 to 1:16 will yield the end point of the titration. In this way errors of dilution may be minimized. The technique of the measurements is in other respects that already described by Hirst and Pickels (9).

## SUMMARY

A detailed study has been made of the nature of the variables inherent in the chicken red cell agglutination test for influenza virus in an effort to obtain a method of measurement of biological activity of sufficient accuracy that it might be employed as a reliable index of chemical purity of preparations of the virus. It was found that the temperature at which the test is conducted has a marked effect on the titer, whereas within the range of pH 6–8 the pH has a negligible effect. It was also found that a variation in results may be encountered due to a variation in the specific behavior of red cells from different chickens and to an instability of the red cells themselves. Preparations of purified influenza virus held at 4°C., on the other hand, were found to be stable with respect to chicken red cell agglutinating activity for several months. This fact, together with the fact that in duplicate measurements upon different samples the accuracy was such that the chances were 19 out of 20 that differences of 8.4 per cent in the mean end points were significant, made it possible to establish a reproducible standard of CCA activity based on a unit weight of purified virus material. As a result, it was possible to devise a standardized procedure for carrying out with high accuracy quantitative measurements of influenza virus.

## BIBLIOGRAPHY

1. Stock, C. C., and Francis, T., Jr., *J. Exp. Med.*, 1940, **71,** 661.
2. Lauffer, M. A., and Miller, G. L., *J. Exp. Med.*, 1944, **79,** 197.
3. Hirst, G. K., *Science*, 1941, **94,** 22.
4. Hirst, G. K., *J. Exp. Med.*, 1942, **75,** 49.
5. Hirst, G. K., *J. Exp. Med.*, 1942, **76,** 195.
6. McClelland, L., and Hare, R., *Canad. Pub. Health J.*, 1941, **32,** 530.
7. Miller, G. L., data to be published.
8. Hirst, G. K., *J. Immunol.*, 1942, **45,** 285.
9. Hirst, G. K., and Pickels, E. G., *J. Immunol.*, 1942, **45,** 273.
10. Stanley, W. M., *J. Exp. Med.*, 1944, **79,** in press.
11. Knight, C. A., data to be published.