AN ABSOLUTE METHOD FOR ASSAY OF VIRUS HEMAGGLUTININS*, ‡, §

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The ability of certain viruses to agglutinate red cells (1) is widely employed as a basis of titration, because of the speed and simplicity of the reaction. Usefulness of the method has been limited, however, by lack of a reliable means of translating hemagglutinating activity into an absolute number of active particles per cubic centimeter, so that values obtained for a single virus can only be expressed in relation to that of some standard suspension, and different viruses cannot be compared at all. Moreover the relationship between hemagglutination and infectiousness is often complex, wide variations in the ratios of these activities being produced by various treatment of virus suspensions (2). An absolute method for determination of the number of hemagglutinating particles would materially increase the usefulness of the virus assay when the two activities are closely associated, and make possible precise analysis of the relationship between infectivity and agglutination in cases in which they are separable. Such a method is described in this communication.

Materials

Red Cells.—Chicken red cells suspended in citrated saline were collected weekly from a slaughter-house, subjected to three cycles of centifugation and washing (1100 R.P.M. for 10 minutes in an International model PR-1 centrifuge), and resuspended in 0.9 per cent saline. Agglutination experiments were carried out in 0.9 per cent saline containing 0.01 M phosphate buffer at the desired pH. A 1.0 per cent suspension of these packed cells contained 5.8×10^7 per cc., as determined by hemocytometer count.

Viruses.—Influenza type A (PR8) and Newcastle disease virus (NDV) were each grown in the allantoic sac of 10 day old embryonated eggs and harvested after 48 hours' incubation at 35°C. Long term storage of these virus suspensions was carried out in a chamber maintained at −35°C.

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Methods of Titrating Virus Activity

Influenza Virus.—Titration of infectivity, *i.e.* ability to multiply in host cells, was carried out in embryonated eggs. The mean infective dose, ID_{50}, was determined by inoculation of five groups of five eggs each with serial dilutions of freshly harvested virus suspensions. After 48 hours of incubation at 35°C., the allantoic fluids of these eggs were tested for the production of virus hemagglutinins. The ID_{50} was calculated by the method of Reed and Muench (3) and converted to infectious particles by the relationship, derived by means of the Poisson distribution formula, 1 ID_{50} = 0.693 infective particles. This method counts only the numbers of fully potent virus particles which are able to carry out the entire reproductive life cycle. Because of the relatively large uncertainty of ID_{50} determinations, the entire procedure was carried out five separate times on five different virus suspensions which were also titrated by the absolute hemagglutination technique. The ratios of the values obtained by these two titration methods carried out on all five suspensions exhibited a standard deviation of 40 per cent. The hemagglutinating titre always exceeded the egg infectivity.

Titration of morphologically identifiable virus particles was effected by electron microscopy. A suspension of influenza virus was purified by three cycles of differential centrifugation, using a Spinco ultracentrifuge for the high speed steps (19000 *g*). The final preparations were concentrated and resuspended in 0.10 M ammonium acetate or distilled water. One aliquot was assayed by the absolute hemagglutination procedure, and another was mixed with a known amount of polystyrene microspheres, sprayed, shadowed, and photographed in the electron microscope in accordance with the procedure of Williams and Backus (4). Excellent drop patterns were obtained in which the virus particles appeared as discrete spherical bodies, with size and shape typical of influenza virus. The virus particles in several single droplets were readily counted in relation to the number of polystyrene spheres. The standard error of these counts amounted to ± 16 per cent.

Finally the influenza virus was also titrated by the pattern test with red cells, using serial dilutions differing by a factor of two, in accordance with the procedure of Salk (5). Activity was expressed in terms of hemagglutinating units, defined as the amount of virus per cubic centimeter just necessary to cause a characteristic "pattern" to form at the bottom of a tube containing a final concentration of 7.2×10^6 cells per cm^3.

Newcastle Disease Virus[1] (*NDV*).—Tiration of infectivity was carried out by plaque count on monolayers of chick embryo cells in accordance with Dulbecco's technique which permits accurate assay of absolute numbers of completely infective particles (6). The two hemagglutination procedures were carried out as in the case of influenza virus, except for the difference in pH in the absolute hemagglutination assay, which will be described. NDV is somewhat more troublesome than influenza because of its greater tendency spontaneously to elute from red cells (7). However, as in the case of influenza virus (8), this reaction does not readily occur unless several virus particles become attached to each red cell. In the absolute hemagglutination assay procedure employed here, this condition is precluded in the tubes from which the final titre is calculated, so that no complications were offered by this phenomenon.

Absolute Hemagglutination Procedure

Optical density–time curves were determined by a method similar to that of Hirst and Pickels (9). To each of a series of optically standardized, 13 × 100 mm. pyrex test tubes was added 6.00 cc. of a buffered saline suspension containing 5.8×10^7 per cm^3. washed chicken red cells and a known serial dilution of the virus to be titrated. At intervals of 10 minutes after addition of the

[1] Thanks are due to Dr. F. B. Bang for supplying the strain of virus employed.

virus the optical density of each tube was read at a wave length of 490 mμ in a Beckman model B spectrophotometer. Stable and reproducible readings were readily obtained when the tubes were simply maintained at room temperature (22°C.) and successively placed in the spectrophotometer by hand. The height of the light beam was 0.85 cm., and its top intercepted the liquid column at a point 3.8 cm. below the meniscus. Thus, the light beam scanned the region in which the active settling process occurs, but missed the area of packed red cells which build up on the bottom, as well as the topmost layer of fluid which loses all its cells and becomes completely clear within 100 to 150 minutes. From data so obtained the number of hemagglutinating particles can be calculated.

Consider the course of events attending the addition of an aliquot of a polyvalent virus (*i.e.*, one which can attach simultaneously to two cells) to a red cell suspension. Let it first be assumed that all the red cells are uniform with respect to their rate of settling. If the erythrocyte concentration is 5×10^7 or greater, attachment of viruses like influenza or Newcastle disease is practically complete within a few minutes (10), a time that is short with respect to the events that follow. The red cells are rendered "sticky" by virtue of the attached virus, which can hold two cells together against the dispersive forces of their thermal energy. In order for clump formation to be initiated, collisions induced through Brownian motion must first occur between the red cells. The rate of formation of two-cell aggregates will then depend on the concentration of the red cells which determines their collision rate according to the Von Smulochowski equation (11); and on the number of agglutinating particles attached to each red cell, which determines the probability that each such collision will actually result in a dimer. Once a dimer is formed, however, its continued growth will depend on another process. Because formation of the dimer results in a decrease of the total surface area exposed to viscous forces of the medium through which the red cells are continually settling, the dimer will fall with a limiting velocity greater than that of single cells. Hence, in falling, the dimer will overtake single cells and, if the ratio of virus to cell has been great enough to make a large proportion of the cell surfaces sticky, the overtaken cells will also be added to the aggregate. The more rapidly new cells are attached, the more swiftly will the resulting aggregate fall; the larger will be the cross-sectional area which it will sweep out as it descends, and hence, the greater the rate of its continued growth. Thus, when the ratio of agglutinating particles to red cells is high, the settling rate will resemble an avalanching process which comes to an end only when the number of single cells left in suspension is so small that the rate of Brownian collision is negligible. Further settling is essentially that of the remaining single cells.

Thus, when the tube contains a large excess of virus over cells, the curve for the time course of the optical density of the central region should display three parts: (*a*) an induction period lasting until Brownian motion collisions have provided sufficient dimers to start the avalanche. For a constant multiplicity of virus to cells this period should depend only on the cell concentration and the viscosity of the medium. (*b*) A period of steadily increasing slope corresponding to the avalanching process itself. (*c*) A section in which the slope stops increasing and starts to decrease, as the number of red cells in the suspension is progressively depleted. Hence, a situation of

this kind should produce a curve with an initial shoulder and a steep inflection point (curves A and B, Fig. 1).

Consider, however, the case in which the initial mixture contains a ratio of virus to cells which is considerably less than one-half. Here an avalanche can never be initiated, because each virus particle can produce only a single dimer which cannot aggregate further. As before, there will be an initial induction period during which

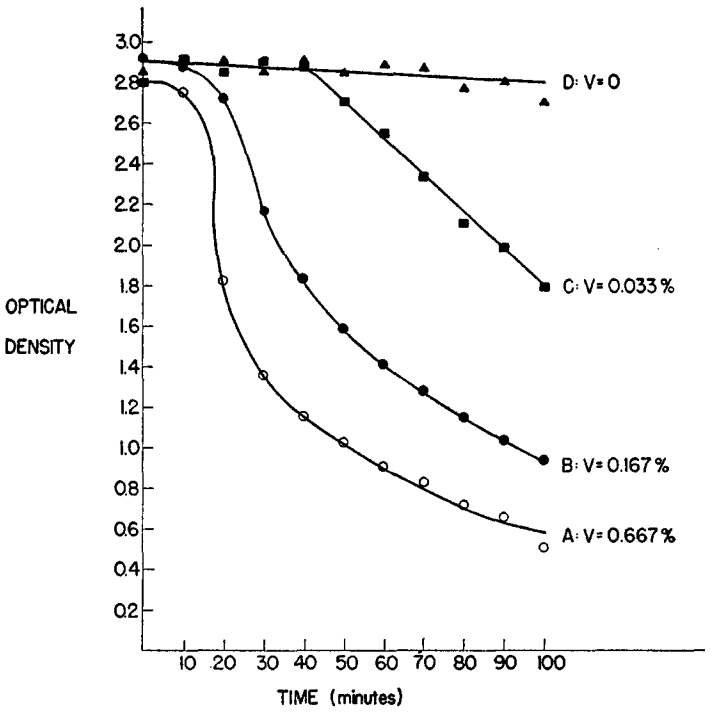

FIG. 1. The time course of the optical density through a region slightly below the center of tubes containing a constant amount of red cells and varying quantities of an influenza virus suspension. As the amount of virus decreases the form of the curve changes from one with a steep inflection (A and B) to a linearly falling optical density (C). Curve D is the control with no virus.

the virus becomes attached to cells and the cell aggregates—in this case only dimers —form. However, these two-cell combinations will settle at a constant rate. The light beam traversing the central region of such a tube will not change in intensity at first because the numbers of particles entering and leaving the scanned region will be equal. However, once the top boundary of the dimer zone enters the illuminated region, the optical density will begin to fall linearly and the rate will remain constant until the boundary reaches the bottom of the light beam, or until a new boundary enters the illuminated region. Thus, the time course of the optical density for this situation will at first be horizontal and then fall linearly (curve C, Fig. 1). We shall call this a "linear settling curve."

Therefore, when serial dilutions of a virus stock are added to red cells, and the optical densities of the resulting suspensions followed, a family of curves should result like those shown in Fig. 1. From a dilution which exhibits a linear settling curve like C (Fig. 1) it is possible to determine the absolute numbers of hemagglutinating particles in the suspension. Since each virus particle in such a suspension is associated with a red cell pair which settles more rapidly than the single cells, determination of the number of these rapidly settling dimers yields the desired value. This determination is complicated slightly by a certain amount of heterogeneity in the settling rates of the single cells, as well as by other factors, which may tend to obscure the formation of sharp boundaries by the monomers and dimers respectively. Thus, before all the dimers have left the illuminated region of the tube, some of the faster settling monomers may have entered. However, once the dimers have all passed out of the field, the slope of the curve from the tube containing virus should be the same as that of the control tube. Hence, the difference in cell densities in these two tubes when the slopes of the optical density–time curves have become equal represents the cells which have been dimerized by the virus. The number of virus particles originally present in the tube is then equal to one-half this difference. The cell densities can be determined by a hemocytometer count on samples removed from the centers of each suspension, or more simply, from the optical density measurements themselves, with the aid of a previously constructed calibration curve. Both methods give essentially identical results. Fig. 2 represents a sample set of curves in which the disappearance of the dimers in the tube with virus is marked by a definite discontinuity in the slope. Sometimes the transition is so gradual that the exact point at which the dimers disappear cannot as easily be identified, but this does not affect the accuracy of the assay. Experience with influenza virus has shown that linear settling curves become parallel to those of the control in the interval approximately between 90 to 150 minutes. Accurately reproducible hemagglutination titres can be obtained by comparing the cell densities in test and control tubes at the level of the light beam, at any time during this interval. The titre of the virus originally added to this tube is then equal to one-half the difference in cell density between this tube and the control without virus, taken at a time during which the two optical density curves are parallel. The titre of the original virus stock can then be calculated from the dilution factor of the aliquot added to the tube displaying the linear settling curve.

A test of the soundness of the foregoing analysis is afforded by the following consideration: Since the presence or absence of a steep inflection in the settling curve has been postulated to depend entirely on the ratio of virus particles to red cells present in the tube, it should be possible to obtain a linear settling curve from an amount of virus that ordinarily produces an inflection like that of curve A of Fig. 1, simply by employing a much denser red cell suspension, so that the ratio of virus to cells will be less than one-half. Fig. 3 illustrates the findings in such an experiment.

It is important that the virus:cell ratio in the settling tube be small, in order to avoid errors due to chance collision of cells containing virus with each other rather than with virus-free cells; or to the occurrence of appreciable numbers of cells to which more than one virus particle has become attached. It can be

demonstrated that such complications can be neglected when the virus-cell ratio is 0.15 or less; *i.e.*, if the assay is made from a straight line settling curve, the optical density of which is approximately 30 per cent less than that of the

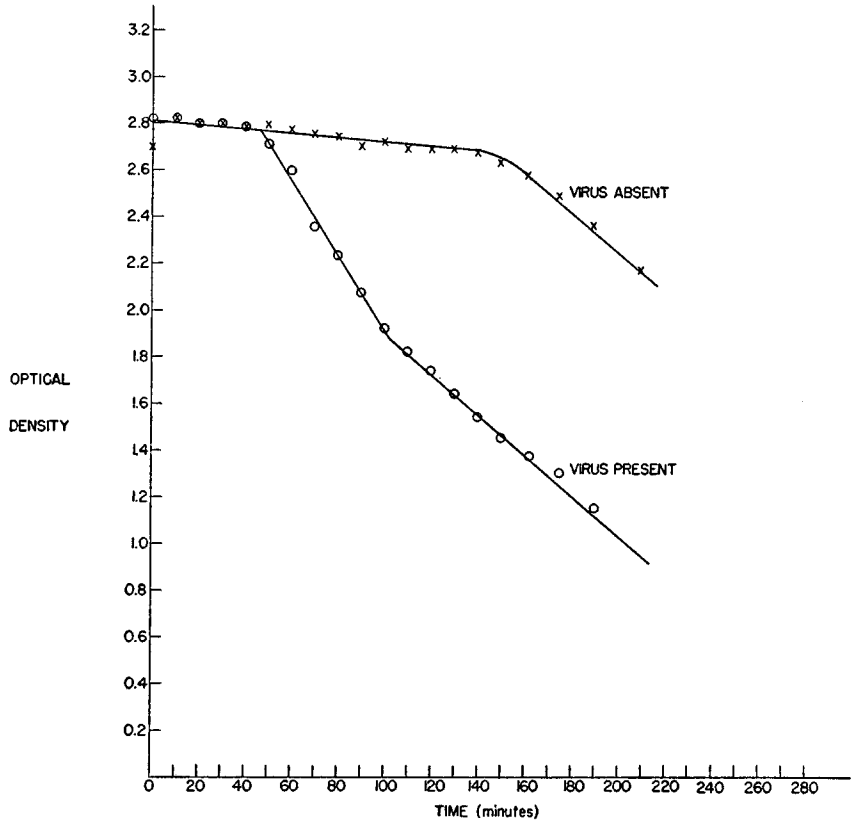

FIG. 2. Demonstration of the end-point determination. The lower curve exhibits the linear type of settling indicating that aggregates larger than dimers have not been formed in appreciable numbers. The break in the curve at 105 minutes marks the time when the upper boundary of the dimers has passed out of the field scanned by the light beam. Thereafter the optical density of the tube containing virus is parallel to that of the control. The difference in cell density in the two tubes at any time during the interval during which the two curves fall at parallel rates is a measure of the number of cells dimerized by the virus particles.

control in the region in which the settling rate has become that characteristic of the monomers.

Table I summarizes the behavior of influenza virus when assayed by the various procedures. It would appear that in the stocks employed one hemagglutinating particle is equivalent, on the average, to 0.41 egg infective particle, and to 1.33 of the particles identifiable by electron microscopy. Also, the ab-

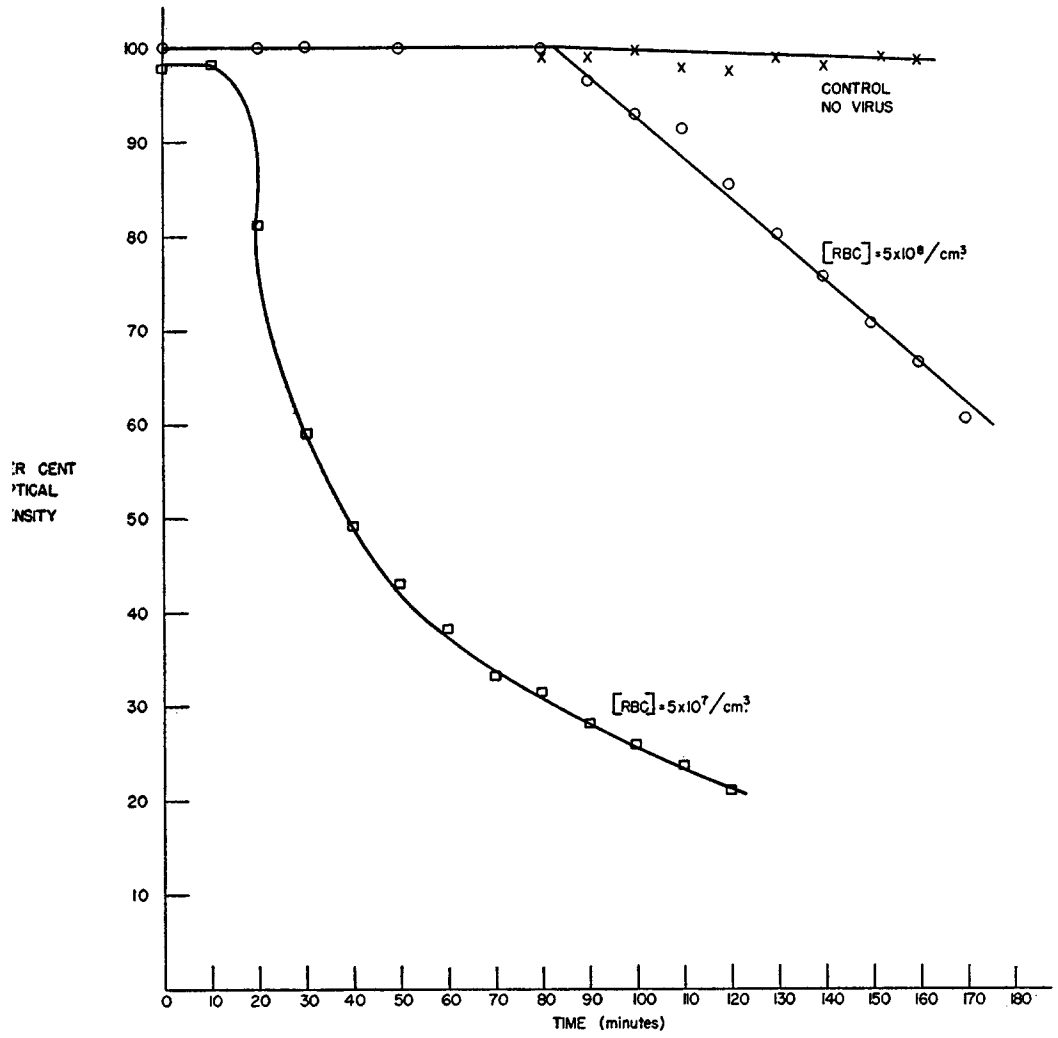

FIG. 3. Demonstration that an amount of virus which produces an avalanching type of settling curve with small amount of red cells will display a linear settling curve if the cells are increased sufficiently. Both ıbes had identical amounts of virus but the red cell concentrations differed by a factor of 10. The optical ensities are expressed in per cent of the value at zero time in each tube.

solute number of particles in one hemagglutinating unit of the pattern procedure is 0.05 times the number of red cells per cubic centimeter in the pattern tubes.

In applying the absolute hemagglutination assay to NDV, it was found that the ability of the virus to precipitate red cells is markedly pH-dependent. At pH 7.00 very little effect is obtained from any virus concentration; at 6.6 good

TABLE I

(*a*) *Comparison of Egg Infectivity and Hemagglutination of Representative Influenza Virus Suspensions*

The egg infectivities were determined on freshly harvested virus preparations.

Egg infectivity (particles/cm.^{3})	Absolute hemagglutination (particles/cm.^{3})	Hemagglutinating units per cm.^{3} by pattern method (using 7.2×10^6 cells/cm.^{3})
1.6×10^9	7.2×10^9	2.4×10^4
1.0×10^9	1.7×10^9	1.2×10^4
1.0×10^9	1.6×10^9	0.30×10^4
0.34×10^9	1.0×10^9	0.20×10^4
0.52×10^9	1.9×10^9	0.60×10^4

(*b*) *Comparison of Electron Microscope Titration and Absolute Hemagglutination of an Influenza Suspension Purified and Concentrated by Differential Centrifugation*

Electron microscope counting (particles/cm.^{3})	Absolute hemagglutination (particles/cm.^{3})
$4.3 \pm 0.6 \times 10^{11}$	3.0×10^{11}
$3.8 \pm 0.6 \times 10^{11}$	

TABLE II

Comparison of 3 Assay Procedures on Two Stocks of Newcastle Disease Virus Prepared and Harvested in Identical Fashion

	Plaque count (infective particles/cm.^{3})	Absolute hemagglutination (hemagglutinating particles per cm.^{3})	Pattern procedure (hemagglutinating units/cm.^{3} for 7.2×10^6 RBC/cm.^{3})
Stock I	7.2×10^8	2.3×10^9	4.1×10^3
Stock II	1.0×10^9	1.8×10^9	8.2×10^3

agglutination curves are obtained, but the potency of the virus reaches a maximum at 6.2 and remains fairly constant down to 5.8. A pH of 6.2 at which the cells are completely stable was selected as the standard for these experiments. Table II presents typical results of the titration of suspensions of NDV virus by the three methods. From these results one agglutinating NDV particle would represent 0.44 chick fibroblast infective particle and the absolute number of particles equivalent to one unit of the pattern procedure is, as with influenza,

equal to 0.05 times the number of red cells per cubic centimeter used in the pattern tubes.

DISCUSSION

Constancy of the ratio between pattern test hemagglutinating activity and egg infectivity of freshly prepared influenza virus was demonstrated by de St. Groth and Cairns (12) who arrived at a value of $10^{6.26}$ ID_{50} doses as equal to one hemagglutinating unit when a suspension of 1.6×10^7 red cells is employed. This corresponds to a value of 1.3×10^6 infectious particles as the hemagglutinating unit for 1.6×10^7 red cells, or an equivalence of 0.08 times the number of red cells per cubic centimeter of absolute particles for each hemagglutinating unit. Values obtained by other workers, summarized by de St. Groth and Cairns, ranged from 0.06 to 0.09. Our value of 0.05, both for influenza and NDV, is somewhat low, but essentially agrees with this figure, considering the uncertainties of the end-point determination of the pattern procedure.

De St. Groth and Cairns also attempted to determine by direct microscopic counting of thousands of cells, the number of dimers formed in the tube corresponding to the end-point of the pattern test. They concluded that a 1:1 relationship exists between the numbers of particles capable of infecting eggs and those which produce microscopically visible cell dimers. Our procedure results in approximately twice as many hemagglutinating as infectious particles in every virus suspension examined, even when infectivity was tested within an hour or two after harvesting the virus. Similarly, the number of microscopically visible cell dimers in our suspensions consistently corresponded to titres approximately half of those obtained by the optical density method. The forces by which a virus holds two red cells together are easily disrupted, even by mild agitation of the suspension. It seems likely, therefore, that an appreciable number of the theoretically possible dimers may fail to form or may be dissociated under the conditions required for the preparation of samples for microscopic examination.

A larger number of cell-agglutinating particles, as compared to those which can carry out all the steps of the life cycle, may well be expected even in fresh virus suspensions, because of the greater sensitivity to inactivation of the more complex reproductive processes. *E. coli* bacteriophage stocks also usually contain approximately two or more times as many particles capable of attachment to and killing of host cells than can form plaques. As in bacteriophage, the plaque count titre of freshly harvested NDV declines rapidly on storage in the refrigerator, but the cell attachment titre remains stable for weeks.

The hemagglutination titre of a suspension provides a measure of the number of particles capable of binding to two red cells. Experiments are currently under way to determine whether the titres so obtained also reflect the ability of these viruses to attach to host cells. Precise experimentation with virus-host systems requires a method for determining the total number of cell-attaching particles

in addition to those capable of multiplication since virus particles which are unable to multiply may, under certain conditions, attach to an already infected cell and contribute to the genetic constitution of the developing progeny (13).

The hemagglutinating titres of virus suspensions used in this study have proved to be stable for periods of several weeks or more. Identical results are obtained from red cells taken from single birds or pooled from large numbers of animals, nor does the time of year or method of blood collection affect the results. The procedure is rapid and convenient. The agreement of the procedure with the numbers of particles obtained with the electron microscope titration method appears to be in the neighborhood of 30 per cent, but the precision of successive determinations is approximately 10 per cent. The degree of uniformity of results with these different methods of titration, based on different properties of the virus, is as good as that obtained with bacteriophage (14).

The close similarity in the standardization values of the hemagglutinating unit of the pattern procedure for NDV and influenza virus suggests that this figure may be usable as a general transforming factor to convert pattern procedure data to absolute hemagglutinating units. This relationship should be tested with other hemagglutinating viruses. The procedure described here may also offer application to the absolute determination of other hemagglutinins, like antibodies.

SUMMARY

A rapid and precise method is described for determination of absolute numbers of hemagglutinating particles present in virus suspensions. The method involves spectrophotometric measurement of the number of red cells dimerized by the virus under conditions in which formation of larger aggregates is precluded.

Results of this procedure are compared with those of titrations based on egg infectivity and the polystyrene microsphere electron micrography for influenza virus; and with plaque count titrations on chick embryo cell monolayers in the case of the virus of Newcastle disease. The agreement is well within the limits expected in view of the different quantities which the various procedures measure.

The conversion factor for transforming titre in hemagglutinating units based on the pattern procedure into absolute number of agglutinating particles is the same for influenza and Newcastle disease virus.

The precipitation of red blood cells by Newcastle disease virus at 23°C. is optimal at pH's between 6.2 and 5.8.

Dr. Frank L. Horsfall, Jr., The Rockefeller Institute for Medical Research, has informed us in a private communication of the independent and concurrent development in his laboratory of a titration procedure similar to that described here.

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