STUDIES ON NEWCASTLE DISEASE VIRUS

I. An Evaluation of the Method of Titration

By F. B. BANG, M.D.

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

(Received for publication, April 6, 1948)

Electron microscope studies of purified preparations of the virus of Newcastle disease have shown that this virus has a filamentous to sperm-like form when dried from saline suspensions (1, 2). If, however, the virus is suspended in water and then dried from this suspension, it has a roughly spherical form, which corresponds to its appearance in the original allantoic fluid (3). An apparent conversion from the spherical form in water to the filamentous form has been brought about by the addition of saline to make a 0.07 to 0.15 M solution (3). The change in shape revealed by electron microscopy was brought about without any detectable change in activity of the suspension. Two questions must be answered before these observations can be assigned any basic significance. (*a*) Could the lack of a detectable change in activity be merely due to inadequacies in the methods of measuring activity? In other words, might we destroy 95 per cent of the virus by the procedure which changes its shape, and not detect this loss of active virus particles? (*b*) Does this change in shape on transfer to saline represent a change which occurs within the solution, or is it merely the result of drying from different solvents? Some experimental evidence against this latter possibility was previously mentioned (3), but more work needs to be done.

We attempt in this paper to evaluate the methods of determining activity (embryo infectivity) and conclude that the changes in shape of the virus cannot be accounted for on the basis of disintegration.

The determination of the 50 per cent mortality or infectivity end-point (4) by means of chick embryos has been in use for a number of years, but very few reports have been made on the accuracy of the method used. Indeed, there is no statistical study available which attempts to estimate the expected probable error with a given number of animals and a given titration end-point slope.

In a limited number of duplicate titrations of Eastern equine encephalomyelitis, a variation of as much as 0.6 log was found between two duplicate titrations (5), using five embryos in each of three dilutions. In a more extensive evaluation of the 50 per cent infectivity measurements for influenza virus in the embryo, Knight (6) found that the chances were 19 out of 20 that differences in end-points of 0.62 logarithmic units were significant.[1] This means that more than 75 per cent of the infectiousness

[1] Five embryos in each dilution.

of a preparation must be destroyed before the odds are as much as 20 to 1 that any decrease at all can be stated to have occurred. The accuracy is, of course, increased by increasing the number of embryos used.

The consistency of results obtained by Sigurdsson (7) when measuring the rate of multiplication of vesicular stomatitis virus in 10-day-old embryos would indicate a roughly similar accuracy for this virus when titered on 7-day-old embryos. Similar consistent results have been obtained in measuring the concentration of equine encephalomyelitis after 23 hours' inoculation (5).

It is, however, at present impossible to predict the accuracy of the method for a new virus infection since the sharpness of the end-point in itself would influence the accuracy of the determination. Quantitative determinations of the sharpness of the end-point, or end-point titration curves are available for only a few animal viruses (8–11) and those not on the embryo.

In a later paper in this series it is shown that infection and death of the embryo are produced by a very few particles of Newcastle virus (12). By use of the theory of random distribution of particles as outlined in the Poisson theory, it is possible to draw a theoretical curve for the expected number of infectious units needed to produce infection. This may be illustrated by two extremes. If one unit is capable of producing infection and a solution containing just enough material to produce infection is diluted $\frac{1}{10}$, then the inoculation to ten times as many embryos with this dilution produces the same total number of infections. If, however, it is necessary to add 1000 infectious units to an embryo to initiate an infection, and a drop of a given suspension contains this much, an equal portion of $\frac{1}{10}$ dilution would rarely have sufficient particles in it to initiate infection. Therefore, the next tenfold dilution would almost always be negative.

These considerations are raised here because they have direct bearing on the accuracy of the method. Fig. 1 shows that the points obtained with small dilutions of the virus best fit a one particle (infectious unit) curve. This does not prove that infection is produced by one unit, but is of practical importance in demonstrating the difficulty in using an end-point other than the 50 per cent end-point.

Materials and Methods

Virus.—The strains of Newcastle disease virus used in this series of studies are as follows: (1) Strain B isolated from a natural outbreak by Dr. F. R. Beaudette of the New Jersey Experiment Station; (2) strain W isolated by us from a natural outbreak of the disease in a flock of chickens in Bound Brook, New Jersey; (3) strain Np isolated by Dr. J. R. Beach in California; (4) Cg179, a laboratory passage virulent strain also isolated by Dr. Beach in California. This strain differs from the others in that it is able to kill 3-month-old chickens in high dilutions following intramuscular inoculation and agglutinates red blood cells poorly. Neutralization tests on embryos with strain W against classical sera furnished by Dr. Brandley demonstrated the immunological similarity. Intramuscular injection of chickens with strain B immunized chickens to Cg179, and agglutination inhibition tests with convalescent sera

from recovered birds inoculated with Cg179 showed the cross-relationship. All four strains are characterized by a filamentous shape when dried from salt solutions (1), produce a high titer of infectivity in the allantoic fluid of embryos, kill the embryo in 2 to 3 days, and often produce a characteristic hemorrhagic pattern which involves the brain and feather follicles.

Method of Titration.—Suspensions of virus were titered after preliminary low speed centrifugation at 5,000 R.P.M. for 5 minutes. Serial tenfold dilutions were made in tubes containing 4.5 cc. of cold buffered saline which had been kept at refrigerator temperature just prior to the titration. Immersion in ice water was omitted because of the stability of this virus. Ten to 12-day-old embryonated eggs opened by making a window in the side of the shell were inoculated with 1 drop of the dilute suspension. All eggs were obtained from the

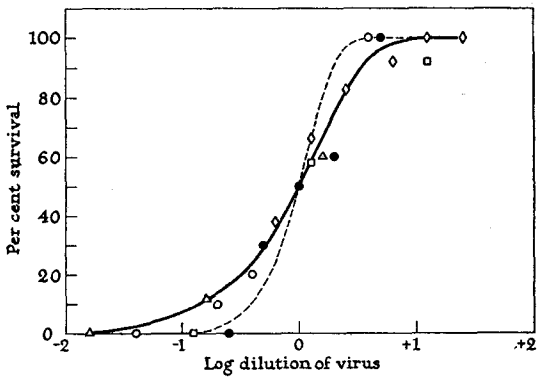

FIG. 1. End-point titration curve of Newcastle virus. Points on this curve were obtained by inoculation of ten embryos at each dilution. A 50 per cent end-point was calculated. The actual per cent of embryos surviving at a given log dilution above or below this calculated end-point is plotted against the log difference between the calculated 50 per cent end-point and the dilution inoculated. Symbols of the same kind represent one experiment. We have combined the results of five experiments to obtain the data. The solid curve is the theoretical curve for infections produced by one infectious unit. The dotted curve is for three units.

Rockefeller Institute stock which has been repeatedly found free of Newcastle disease as shown by the lack of immunity demonstrated by red cell agglutination inhibition tests and inoculation of virulent strains of the virus.

Embryos were usually incubated at 35° after inoculation (see below) and the occasional embryo dying within the first 12 to 18 hours was considered as killed by trauma and was discarded. With dilute suspensions of virus, death of the embryo begins about 2½ days after inoculation and may continue through 3 or 4 days. Incubation at higher temperatures produces more rapid multiplication of the virus and consequently a more rapid death. All surviving embryos were kept for 6 days after inoculation before discarding.

Smears were made as routine from dead embryos for microscopic examination to eliminate the occasional death occurring from accidental contamination. Previous work with gonococcus prophylaxis in embryos (13) has shown that this method picks up the great majority of such infections. However in critical experiments, blood plate cultures were also made. Since the virus of Newcastle disease agglutinates red cells, we usually checked the ability of the allantoic fluid from dead embryos used in a titration to do this. There are, however, several difficulties involved. First, concentrated suspensions of unpurified virus often show a prozone effect—possibly due to an inhibitor in the allantoic fluid. Therefore, it is necessary

to check two or three tenfold dilutions of the allantoic fluid before stating that it is negative. Secondly, different strains vary in their ability to agglutinate. Strain Cg179, our virulent laboratory strain, is a poor agglutinator producing irregular agglutination of chicken red cells only up to dilutions of 1/100 or so. Thirdly, embryos inoculated on the chorioallantoic membrane do not always develop sufficient virus in the allantoic fluid to agglutinate red cells. Thus, we cannot consider deaths which occurred at the right time and in the absence of bacteria, as not being due to Newcastle virus if they did not show red cell agglutination. However, several of the five embryos in a dilution would usually, if they died, have sufficient virus to agglutinate chicken red cells, and we could therefore conclude that the virus was present in that particular dilution.

It is well to emphasize that similar accuracy to that obtained here cannot be expected if the source of embryos is not from a clean flock of chickens, and if the above considerations are not taken into account. Particular care should be taken to demonstrate that deaths from other causes not following inoculation are not occurring in the eggs between the 10th and 18th days of incubation. This may be a common source of error when using eggs bought on the open market.

TABLE I

Duplicate Titrations of Various Suspensions

50 per cent end-point mortality. 3 dilutions—5 embryos each dilution

Virus	Duplicate titers		
	Titer 1	Titer 2	Difference
B—allantoic fluid	$10^{-7.4}$	$10^{-7.2}$	−0.2
B— " "	$10^{-8.7}$	$10^{-8.6}$	−0.1
Cg179—" "	$10^{-8.5}$	$10^{-8.4}$	−0.1
Cg179—" "	$10^{-7.8}$	$10^{-8.0}$	+0.2
B— " "	$10^{-8.7}$	$10^{-8.5}$	−0.2
B—embryo suspension	$10^{-6.2}$	$10^{-5.8}$	−0.4
B—purified preparation	$10^{-7.6}$	$10^{-7.8}$	−0.2
Same titered in water		$10^{-7.8}$	

RESULTS

Table I presents the results of duplicate titrations on different preparations carried out during the course of our investigation. None of these seven tests differed from the duplicate by more than 0.4 log. We therefore believe that a difference of 0.6 log is probably significant and of 1.0 log almost certainly significant. This would mean that we probably could detect a loss of 75 per cent of activity and almost certainly a loss of 90 per cent activity. A variety of other factors might be of significance in titering Newcastle virus in the embryo and we here present data bearing on some of them.

Temperature.—It has been shown for a number of viruses that the optimum temperature range is fairly narrow. For influenza it is about 35–36°C. This suggests that titrations might better be carried out at that optimum. How-

ever, the optimum temperature range for growth of Newcastle virus seems to be much greater (14). The effect of different temperatures of incubation after inoculation of the embryos with the definitive dilutions was studied by performing titration as usual, but ten instead of five embryos were inoculated at each dilution. One-half of the embryos in each dilution were incubated at one temperature and the other half at another. The 50 per cent end-points were calculated separately and are compared in Table II.

TABLE II

Effect of Temperature of Incubation on Titer Obtained from Same Preparation of Virus

Age of embryo used	Temperature of incubation	
	35°C.	39°C.
10 days	$10^{-6.5}$	$10^{-6.3}$
14 days	$10^{-6.5}$	$10^{-6.7}$

TABLE III

Effect of Route of Inoculation on Titration Results

Virus	On chorioallantoic membrane	In allantoic sac	In amniotic sac	In yolk
Cg179	$10^{-8.2}$	$10^{-7.8}$		
Cg179 6-day-old fluid	$10^{-7.8}$	$10^{-8.2}$	$10^{-8.2}$	
B strain suspension embryo	$10^{-6.7}$	$10^{-7.2}$		
B strain allantoic fluid	$10^{-9.5}$	$10^{-9.5}$		
B strain concentrated	$10^{-9.2}$			$10^{-9.0}$
B strain 46 hr. growth	$10^{-8.5}$			$10^{-9.3}$
Cg179 stock	$10^{-7.5}$			$10^{-6.8}$

Route of Inoculation.—With different strains of mumps it is necessary to introduce the inoculum by different routes to get the highest degree of takes (15). The virus of swine influenza is likely to kill the embryo if injected into the allantoic sac (16), but if inoculated by itself on the chorioallantoic membrane, relatively few of the embryos die (17); many throw off the infection and survive. It might therefore be expected that the route of inoculation would play some rôle in determining the 50 per cent end-point. A number of tests were made, again by inoculating the same dilution fluid by different routes into several series of embryos.

Table III shows the results from a series of such tests.

The inoculation on the chorioallantoic membrane was performed by first lowering the membrane through an artificial window in the side, then dropping 1 drop of the dilution tested on the membrane, and sealing with scotch tape. Allantoic inoculation was usually performed

by injection of about 0.05 cc. through a small hole in the otherwise intact egg with a short hypodermic needle. Amniotic inoculation was by direct inoculation with the visualization of the amniotic sac through an artificial window in the side. A pair of fine forceps held the edge of the amniotic membrane. Yolk sac inoculation was by a long needle (1¼ inches) inserted through the blunt end of the egg.

Table III shows that there is no consistent difference between the methods of inoculation and that they all yield comparable results. This is in agreement with other recent reports (18). We continue however to prefer for this virus the inoculation on the chorioallantoic membrane because we believe that this method allows a more careful check of accidental deaths and gives slightly more consistent results.

Effect of Age of Embryo.—The effect of the age of the embryo used in measuring virus activity was studied by inoculating the same dilutions on two sets of

TABLE IV

Influence of Age of Embryo Used in Titration on the Calculated 50 Per Cent End-Point

Experiment No.	Age, *days*					
	10	11	13	14	15	16
1	$10^{-8.3}$				$10^{-8.3}$	
2	$10^{-6.5}$			$10^{-6.5}$		
3		$10^{-7.8}$		$10^{-7.5}$		
4	$10^{-9.2}$		$10^{-8.3}$			$10^{-7.8}$

embryos (chorioallantoic membrane) of different ages. The results of this are shown in Table IV. All inoculations included in this table were with strain B.

Three of the four experiments failed to show any effect of the age of the inoculated embryo. The fourth did show a probably significant effect of age. We, therefore, prefer 10- or 11-day embryos for titrations, but 12-day embryos may also be used, and when used in these experiments their use has been so specified.

Effect of Red Blood Cells.—Several of the viruses which agglutinate red blood cells are absorbed on the chick red cells in large amounts. It might, therefore, be expected that accidental contamination of the fluid with red blood cells during harvesting would considerably affect the titer obtained. However, actual tests indicate that at room temperature a minimum amount of virus is absorbed. In order to give a maximum chance for absorption the following experiments were done with the B strain which is a good agglutinator. The allantoic fluid virus was diluted to $\frac{1}{100}$ and to this a sufficient amount of red blood cells to make a 1 per cent suspension was added. Since agglutination is better at ice box temperature (19), the virus suspension with red cells was placed in the refrigerator for varying periods of time. The chicken red cells

were then spun down and the supernatant fluid titered for virus activity by inoculation of serial tenfold dilutions. Controls were similarly treated except for the absence of red blood cells. The results are shown in Table V.

There is a consistent reduction in the embryo infectivity. This reduction is only occasionally statistically significant. These results would agree with those of Florman in indicating absorption of the virus on the red cell, but indicate clearly that a few red cells gaining entrance into the allantoic fluid when harvesting are not an important source of error.

TABLE V

Effect of Addition of 1 Per Cent Chicken Red Blood Cell Suspension to 1/100 Dilution of Virus

Allantoic fluid virus	Time after red cells added, *min.*						Control
	Immediately	7	20	30	45	60	
Diluted 1/100.....					$10^{-8.5}$		$10^{-8.7}$
" 1/100.....				$10^{-7.0}$			$10^{-7.5}$
Purified by centrifugation. Dilution 1/10..	$10^{-9.0}$	$10^{-8.5}$	$10^{-8.8}$			$10^{-9.2}$	$10^{-9.2}$

SUMMARY

The application of the 50 per cent embryo mortality to a study of the virus of Newcastle is described. It has been evaluated by a series of duplicate titrations of the same sample of virus. In seven such titrations the largest difference between the two was $10^{-0.4}$. It is therefore believed that a difference of 0.6 log is probably significant and of 1.0 log almost certainly significant. This would mean that we can almost certainly detect a loss of 90 per cent of activity.

Neither temperature of incubation nor route of inoculation in the test embryos had consistent effect on the measurement of virus activity. The effect of increasing age of the incubated embryo, from 10 days up to 16 days, is slight and inconsistent. The addition of chicken red blood cells to a dilution of virus may lower the titer of the preparation, but the change is not sufficient to be of importance in the routine handling of the virus.

BIBLIOGRAPHY

1. Bang, F. B., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63,** 5.
2. Cunha, R., Weil, M. L., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1947, **55,** 69.
3. Bang, F, B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64,** 135.
4. Reed, T. J., and Muench, H., *Am. J. Hyg.*, 1928, **27,** 493.
5. Bang, F. B., *J. Exp. Med.*, 1943, **77,** 337.
6. Knight, C. A., *J. Exp. Med.*, 1944, **79,** 487.

7. Sigurdsson, B., *J. Exp. Med.*, 1943, **78,** 17.
8. Parker, R. F., Bronson, L. H., and Green, R. H., *J. Exp. Med.*, 1941, **74,** 263.
9. Parker, R. F., *J. Exp. Med.*, 1940, **71,** 439.
10. Bryan, W. R., and Beard, J. W., *J. Infect. Dis.*, 1939, **65,** 306.
11. Morgan, I. M., *Am. J. Hyg.*, 1947, **45,** 372.
12. Bang, F. B., *J. Exp. Med.*, 1948, **88,** 251.
13. Bang, F. B., *Am. J. Syph., Gonor., and Vener. Dis.*, 1943, **27,** 716.
14. Bang, F. B., *J. Exp. Med.*, 1948, **88,** 241.
15. Habel, K., *Pub. Health Rep., U. S. P. H. S.*, 1945, **60,** 201.
16. Sigurdsson, B., *J. Immunol.*, 1944, **48,** 39.
17. Bang, F. B., *J. Exp. Med.*, 1943, **77,** 7.
18. Hanson, R. P., Winslow, N. S., and Brandly, C. A., *Am. J. Vet. Research,* 1947, **8,** 416.
19. Florman, A. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64,** 458.