TITRATION OF INFLUENZA VIRUS IN CHICK EMBRYOS*

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Two methods are currently used for quantitatively titrating the infectivity of influenza viruses. One of these is the mouse test (1, 2) and the other is the more recent chick embryo titration employing chicken red cell agglutination as a criterion of infection (3). The significance of results obtained by the mouse titration employing five mice per test dilution was recently established by a statistical study (2), but comparable data have not been reported for the embryo test. It appeared probable from the data obtained in preliminary experiments and also from statistical considerations that the use of ten embryos per dilution would result in more precise and reproducible end points than had been obtained in the titrations with five mice. This has indeed proven to be the case, as will be shown in the present report. In addition, the results obtained in some comparable titrations in mice and in embryos are given.

Materials and Methods

Preparation of Material.—Infective allantoic fluid containing the PR8 strain of influenza virus was centrifuged for 20 to 30 minutes in a high-speed centrifuge (4). The pellets thus obtained were suspended in 0.1 M phosphate buffer at pH 7 (5) and centrifuged for 5 minutes at low speed in a clinical centrifuge to remove insoluble matter. The supernatant solution from this centrifugation was used as a source of virus. The amount of virus in the solution was estimated from nitrogen analyses made by the Nessler method (6). A fresh solution of virus, prepared in the manner just described, was employed in each of 6 titrations. Serial hundredfold dilutions of virus were made in sterile 0.1 M phosphate buffer at pH 7 until the lowest dilution of the range to be tested was reached. From this point, serial tenfold dilutions were made to cover the 5 successive dilutions in which the end point was expected to occur. A separate series of dilutions was made for each replicate titration.

Titrations in Chick Embryos.—Embryonated eggs which had been incubated for 10 days at 39°C. were used in all titrations. The eggs were carefully candled and marked with a pencil at the base of the air sac and directly over the embryo. Eggs which showed any signs of abnormality were rejected. The eggs were placed upright on racks accommodating 40 eggs and sprayed with 70 per cent alcohol. Holes were punched through the shells near the pencil marks with a small hand drill which was

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TABLE I

Results of Replicate Titrations of the PR8 Strain of Influenza Virus in Chick Embryos

Test No.	Concentration of inoculum, *gm. per ml.*					50 per cent end points (negative exponents of 10)
	10^{-11}	10^{-12}	10^{-13}	10^{-14}	10^{-15}	
	9/10*	7/10	1/10	0/9		12.3
	10/10	7/10	0/8	0/10		12.3
1	10/10	5/10	2/9	0/9		12.2
	9/10	5/10	1/10	0/10		12.0
	9/10	7/10	0/9	0/7		12.2
	10/10	9/10	10/10	8/10	0/10	14.3
	10/10	10/10	9/10	7/10	0/10	14.2
2	10/10	10/10	10/10	7/10	0/10	14.3
	10/10	10/10	8/10	7/10	2/10	14.3
	10/10	10/10	9/10	6/10	0/10	14.1
	10/10	10/10	7/10	5/9	1/10	13.9
	10/10	10/10	6/10	7/10	0/10	14.0
3	10/10	10/10	8/10	6/10	0/10	14.0
	10/10	10/10	7/10	6/10	1/10	14.0
	10/10	10/10	7/10	5/10	0/10	13.7
	10/10	8/10	6/10	2/10	0/10	13.2
	10/10	10/10	7/10	3/10	0/10	13.5
4	10/10	10/10	7/10	0/10	0/10	13.3
	10/10	10/10	6/10	3/10	0/10	13.4
	10/10	10/10	4/10	2/10	0/10	13.0
	10/10	10/10	10/10	5/10	1/10	14.1
	10/10	10/10	9/9	6/10	0/10	14.2
5	10/10	10/10	10/10	7/10	0/10	14.3
	10/10	10/10	10/10	6/10	0/10	14.2
	10/10	10/10	10/10	5/10	1/10	14.1
	8/10	10/10	8/10	3/10	0/10	13.5
	10/10	10/10	6/10	1/10	1/10	13.3
6	9/10	10/10	8/10	0/10	0/10	13.3
	10/10	10/10	9/10	2/10	0/10	13.6
	9/10	9/10	8/10	1/10	0/10	13.3

* Numerators indicate the number of embryos that yielded allantoic fluid containing demonstrable hemagglutinins. The denominators indicate the number of embryos successfully harvested. Occasional yolky or hemorrhagic fluids were excluded from the test.

dipped occasionally in 70 per cent alcohol. Ten eggs were employed for each dilution in each replicate titration. An arbitrarily chosen volume of 0.8 ml. of solution was inoculated into each egg.[1] After inoculation the eggs were sealed with colored collodion

[1] Subsequent tests have shown that 0.1 or 0.2 ml. of inocula yield entirely satisfactory results with PR8 and other strains of influenza virus.

and incubated at 36°C. for 40 to 48 hours. The eggs were next chilled for 5 to 15 hours at 4°C. and the allantoic fluid from each egg was tested for hemagglutinins with a 1.5 per cent suspension of chicken red cells (3). The numbers of positive and negative tests for hemagglutinins after 30 to 45 minutes were recorded and from these results 50 per cent infectivity end points were calculated by the method of Reed and Muench (7). The data obtained are presented in Table I.

Mouse Titrations.—The mouse titrations were performed as described by Lauffer and Miller (2) with the exception that ten mice were employed for each dilution tested. The method of weighted end points was used in expressing results.

Reproducibility of Titrations.—The variance, V, of the end point for each set of replicate titrations was calculated by use of methods of small sample statistics as described by Lauffer and Miller (2). From the average variance of the end points,

TABLE II

Variances and Standard Deviation of End Points Obtained in Titrations of Influenza Virus in Chick Embryos

Test No.	ΣX^2	$\frac{(\Sigma X)^2}{N}$	Difference	$V = \frac{\text{Difference}}{N-1}$ Variance	Standard deviation
1	744.260	744.200	0.060	0.015	
2	1013.920	1013.888	0.032	0.008	
3	968.900	968.832	0.068	0.017	0.132
4	881.940	881.792	0.148	0.037	
5	1005.390	1005.362	0.028	0.007	
6	897.880	897.800	0.080	0.020	

the standard deviation of the distribution of end points was calculated. These data are shown in Table II. A standard deviation of 0.132 was obtained as compared with the values of 0.237 and 0.262 calculated by Lauffer and Miller for their weighted end point and Horsfall's mortality end point mouse titrations, respectively. Since these standard deviations are measures of the reproducibilities of the end point determinations, it is apparent that the chick embryo titration employing ten eggs per dilution is capable of greater reproducibility than the mouse titration employing five or six mice per dilution. However, a calculated value of 0.225 was obtained for the standard deviation of the distribution of end points if five embryos were employed.[2] This

[2] The data from the first three tests of Table I were distributed in groups of five embryos by the following sampling procedure. Blue marbles were considered to represent embryos whose allantoic fluids contained demonstrable hemagglutinins and red marbles were considered symbolic of negative tests. Appropriate numbers of positive and negative marbles were mixed in a box and five successive marbles were withdrawn. The numbers of positive and negative marbles among those withdrawn and those left behind were recorded. In this manner, the data for ten embryos were in each case converted into data for two groups of five embryos. End points were calculated as previously described and from these values the variance and standard deviation of the distribution of end points were calculated.

figure is very close to the best value obtained in the mouse titrations and suggests that the reproducibilities of end points in the mouse and embryo titrations are about the same when five animals per dilution are used.

Statistical Significance of Differences between End Points.—The standard deviation of successively determined end points is $\sqrt{2}$ times the standard deviation of the distribution of end points for a given method of titration. This value in the case of the ten-embryo titration is 0.187, and from it by use of appropriate tables (8) one can calculate the probability of significance for various end point differences. Some calculations of this sort are presented in Table III. In statistical analysis, deviations exceeding twice the standard deviation are formally regarded as significant. Hence, a difference of 0.37 or greater between the logarithms of the end points of two preparations of virus titrated in chick embryos, using ten embryos per dilution, is significant, or conversely, the chances are about 19 out of 20 that the two preparations are not identical. Further, the comparison of mouse titration with embryo titrations presented in Table III indicates that the mouse titration employing five mice per dilution

TABLE III

End Point Difference Required for Various Levels of Probability of Significance

Probability	End point difference required		
	Mouse titration*	Embryo titration	
	5 mice	5 embryos	10 embryos
0.90	0.61	0.52	0.31
0.95	0.73	0.62	0.37
0.99	0.95	0.82	0.48
0.999	1.21	1.05	0.62

* From data of Lauffer and Miller (2).

can be expected to fail to detect a fourfold difference in virus concentration about one time in 10, that the embryo titration using five embryos per dilution would register a similar failure one time in 20, and that the embryo titration employing ten embryos per dilution would fail to detect a fourfold difference only about once in a thousand trials. In general, the reproducibility of end points for the ten-embryo titration appears to be about twice as great as for the mouse or embryo tests employing five animals per dilution.

Comparative Sensitivity of the Mouse and Chick Embryo Tests to Different Preparations of PR8 Virus

In studies concerned with the pH stability of virus, the effect of chemicals upon the virus, the efficacy of various preparative treatments, or the association of virus activity with various sizes of material, one frequently encounters cases in which either the amount of virus is small in comparison with the amount of non-viral material present or the quantity of active virus is only a tiny fraction of the amount of inactive virus. Hirst showed that in testing small amounts of virus in allantoic fluids the embryo titration is from ten to several

hundred times more sensitive than the mouse test (3). Henle and Henle found that the infectivity of virus in allantoic fluids could be appreciably reduced by the addition of similar fluids in which the virus had been partially inactivated by treatment with heat or ultraviolet light, although it was not demonstrated whether such inhibition is greater in embryos or in mice (9). Stanley observed, in a comparison of the specific virus activities of two purified preparations of high molecular weight materials obtained from F 12 allantoic fluid, that the apparent difference in infectivities was of much smaller magnitude in the case of the mouse test than in the embryo titration when five animals were used to test each dilution (10). In general, these observations suggested the advisability of investigating the comparative sensitivities of the mouse and chick embryo tests to purified preparations of virus and the comparative effect of inactive virus on the two tests. Both of these points were investigated simultaneously in parallel titrations of three pairs of virus preparations. In order to increase the accuracy of the observations, ten animals per dilution were employed.

In the first experiment a purified preparation of PR8 virus known to have fairly high activity was compared with an allantoic fluid which was known to possess approximately its full initial activity. These are preparations 1 and 2, respectively, of Experiment A in Table IV. It can be seen that the 50 per cent end points of the mouse titrations are at concentrations of virus of about 10^4 times as great as those at the 50 per cent end points of the corresponding embryo titrations.[3] Despite this fact, it is apparent that in the case of these essentially fully active preparations the difference between the activities of preparations 1 and 2 is about the same order of magnitude whether judged by the mouse test or by the embryo titration.

In Experiment B, two purified preparations were compared, one of which was known to have lost about 50 per cent of its original activity and the other of which was known to have lost approximately 99.99 per cent of its initial potency. Again the mouse titration end points occurred at concentrations of virus about 10^4 to 10^5 times those at which the embryo end points were found. With regard to the relative activities of preparations 1 and 2, however, one can obtain two different answers depending upon whether the mouse titrations or the embryo titrations are used as criteria. The mouse titrations indicate that preparation 1 is about 500 times as active as preparation 2, but the embryo tests indicate that 1 is approximately 8000 times as active as 2. If one can assume that the accuracy of the ten-mouse titration corresponds with that of the ten-embryo test, the probability that such a discrepancy could be due to chance alone is negligible.

Finally, in Experiment C, a freshly obtained purified preparation (No. 1)

[3] Since the amount of inoculum used per mouse was about one-tenth that used per embryo, it is necessary to increase by tenfold the mouse end points in Table IV in order to compare them directly with the embryo end points.

TABLE IV

A Comparison of the Chick Embryo Titration with the Mouse Titration in Determining the Infectivity of Various Preparations of PR8 Virus

Experiment	Preparation No.	Embryo titration—red cell tests									50 per cent end point	Mouse test—weighted end points							50 per cent end point
		10^{-7}*	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	10^{-13}	10^{-14}	10^{-15}		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	
A	1						10/10‡	8/10	3/10	0/10	$10^{-13.6}$					$D_5,D_6,D_5,D_6,$ $D_6,D_6,D_7,$ D_7,D_8,D_8	$D_8,D_8,D_8,2,$ $2,2,2,2,2$	All 0	$10^{-8.2}$
	2	10/10	10/10	1/9	0/10						$10^{-8.6}$	$D_4,D_4,D_4,D_5,$ $D_5,D_5,D_6,D_6,$ D_7,D_7	$D_4,D_4,1,1,1,$ $1,1,0,0,0$	All 0	All 0				$10^{-8.7}$
B	1					10/10	10/10	6/10	0/10		$10^{-12.2}$				$D_5,D_5,D_5,D_6,$ $D_6,D_6,D_7,$ D_8,D_8,D_{10}	$D_8,D_8,3,3,3,$ $2,2,2,2,2$	$1,0,0,0,0,0,$ $0,0,0,0$		$10^{-7.3}$
	2	7/7	10/10	6/9	0/10						$10^{-9.3}$		$D_4,D_5,D_5,D_6,$ $D_6,D_6,D_7,D_8,$ $3,3$	$1,1,1,1,1,1,1,$ $1,0,0,0$	All 0				$10^{-4.6}$
C	1						10/10	10/10	6/10	0/10	$10^{-14.2}$					$D_4,D_5,D_5,$ $D_5,D_5,D_5,$ $D_6,D_6,D_7,2$	$D_8,D_9,2,1,1,$ $1,1,1,1,1$	$1,1,1,1,0,$ $0,0,0,0,0$	$10^{-7.9}$
	2		10/10	4/10	1/10	0/10					$10^{-8.9}$	$D_6,1,1,\pm,\pm,$ $\pm,\pm,0,0,0$	$D_8,D_8,D_8,1,1,$ $1,1,0,0,0$	$1,1,1,\pm,\pm,$ $\pm,\pm,0,0,0$	All 0				?

* The figures represent grams of material per milliliter of inoculum, except in the case of the allantoic fluid, preparation 2 in Experiment A, in which case the numbers represent dilution of the fluid.

‡ The symbols used for recording results are the same as in Table I.

was compared with a purified preparation which had been about 99.999 per cent inactivated by storage for several weeks at pH 10. Satisfactory end points were obtained for both preparations by the embryo titration, but in the case of preparation 2 it was impossible to calculate a satisfactory end point from the data obtained in the mouse test. It is apparent from the data that a tenfold dilution of preparation 2 resulted in more deaths and greater lesions in mice than were obtained when the preparation was inoculated at a full strength of 1 mg. per ml. This result strikingly illustrates the importance of the inhibitory effect of inactive virus upon the mouse titration. This inhibition undoubtedly affects the embryo titration also when proper proportions of active and inactive virus are obtained, but in the present embryo titrations there was no appreciable evidence for the effect up to and including the 99.999 per cent inactivation point. On the other hand, the mouse titrations, as judged by comparison with the embryo titrations, are susceptible to inhibitory influences possibly even in the case of so called fully active preparations of virus, and this suceptibility becomes progressively greater with increased percentages of inactive virus until the data obtained are no longer capable of quantitative evaluation.

DISCUSSION

The data presented in Table III provide a basis for selection of an appropriate number of embryos for the titration of PR8 virus preparations when a definite accuracy is desired. Using ten embryos per dilution, one can expect frequently to detect a twofold and almost always a threefold difference in virus activity. With five embryos or five mice, one can scarcely expect to detect with any certainty differences less than four- or fivefold. Thus, the results demonstrate that in accordance with theory the ten-embryo titration is to be preferred to the five-embryo or five-mouse test for most quantitative determinations of virus activity.

One might expect from the data in Table III that the reproducibility of end points in titrations employing ten mice per test dilution would correspond approximately with that found for the ten-embryo titration, although this has not been demonstrated. Both tests are considered to be accurate and sensitive methods for the titration of influenza viruses and neutralizing antibodies, and it appears that the precision and reproducibility of end points obtained by the two methods are about the same. However, the chick embryo titration possesses certain advantages over the mouse test. Tests can be completed in 2 days rather than in 10; the embryo test is sensitive to smaller amounts of virus and conversely, less susceptible to inhibition by inactive virus; the embryo titration can be used with non-mouse adapted strains, such as those which have been directly isolated from human throat washings by inoculation in chick embryos (3, 11, 12); extensive titrations can be made with less equip-

ment; embryos are much less subject to natural diseases than are mice (13); and since only a fraction of the allantoic fluid from each embryo used in a titration is required for the agglutination test, the bulk of the fluid can be recovered and conveniently used as a source of virus. In the mouse test, less attention need be paid to sterility than in the embryo test. In most cases, however, this is a doubtful advantage, for in moderately contaminated solutions the greater sensitivity of the embryo test permits the contaminants to be diluted out and in grossly contaminated solutions the results in either test can be considered questionable. The fact that the results of the embryo titration parallel those obtained in the mouse test when active preparations of virus are titered indicates that the mouse titrations possess no advantage over the embryo tests by virtue of the closer resemblance of the infection in their case to a presumably more natural disease.

The finding that the embryo titration is 10^4 to 10^5 times as sensitive to virus as the mouse titration when used to test purified preparations of virus may make it more useful in certain instances, although in routine titrations of active virus, this factor is probably insignificant. On the other hand, the smaller susceptibility of embryos to the inhibitory effect of inactive virus makes it appear desirable to employ the embryo titration rather than the mouse titration in testing virus preparations in which, due to chemical or physical manipulations, an appreciable amount of inactivation may have occurred. This factor may also prove of importance in the titration of neutralizing antibodies.

Since no abnormalities were observed in titrations of allantoic fluids containing several strains of virus (3), it seems reasonable to expect that the data obtained in the present titrations of purified PR8 virus can serve as a guide in evaluating tests made on preparations of other strains of influenza virus. The application of the method should be further broadened by its simplicity, for it was found that the results obtained by a person who had not previously performed such a titration (Experiment 6, Table I) differed only slightly from those obtained by an experienced operator.

SUMMARY

A study was made to establish the reproducibility of end points obtained in the titration of influenza viruses in chick embryos. Six tests were performed, each composed of five replicate titrations of a purified preparation of the PR8 strain of influenza virus. The data from these titrations were subjected to statistical analysis which revealed that the chances are 19 out of 20 that differences in end points of 0.37 and 0.62 logarithmic units are significant in titrations employing ten embryos and five embryos per dilution, respectively. Additional simultaneous titrations in embryos and in mice showed that chick embryos are sensitive to considerably smaller amounts of virus than are mice, and that the mouse titration is adversely affected by inactive virus under conditions which are without apparent influence on the embryo titration.

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