THE MEMBRANE PIECE TECHNIQUE FOR *IN VITRO* INFECTIVITY TITRATIONS OF INFLUENZA VIRUS

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(With 2 Figures in the Text)

INTRODUCTION

The infectivity of an influenza virus preparation has commonly been measured by determining the 50% infectivity dose in fertile eggs. This method can yield quite consistent and reliable results (Knight, 1944; von Magnus, 1951*a*), but is laborious and expensive.

Fulton devised an ingenious test (Fulton & Armitage, 1951) in which small pieces of chorio-allantoic membrane were substituted for whole eggs. The membrane was removed from an egg and washed, and then while floating in a Petri dish of saline, it was cut into about twenty-five pieces of approximately 12×12 mm. One piece was placed in each of a series of cups in a special perspex tray, together with 1 ml. of a glucose-buffered salt solution and two drops of the appropriate virus dilution. The tray was rocked in an incubator at 36° C. After 60 hr., the pieces of membrane were removed, and the fluid in each depression was tested for haemagglutinins. From the proportion of fluids containing haemagglutinins at each virus dilution, a 50% infectivity titre was calculated. This method gave results consistent with those of egg titrations, and was much more economical. But unfortunately cutting the chorio-allantoic membrane pieces proved laborious, and there was little saving in time.

In this laboratory, a technique for infectivity tests has been developed which is derived from that of Fulton, but allows rapid and large scale working and uses ordinary laboratory equipment. In particular, the chorio-allantoic membranes are cut into suitable pieces while still lining the inside of the shells. About thirty pieces of shell with the chorio-allantoic membrane still attached (*membrane pieces*) can be cut rapidly from each egg, and are used in this form in titrations. The method has proved reliable and economical in more than 12 months of use, and is described in detail in this paper.

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MATERIALS

(1) *Solutions*

(*a*) Buffered modified glucosol, pH 6·8 (Fulton & Armitage, 1951). Equal volumes of sterile modified glucosol and of phosphate buffer, pH 6·8 are mixed. It has been found convenient to make up bulk concentrated solutions as follows:

(i) Modified glucosol (ten times concentrated solution)

NaCl	160 gm.
$CaCl_2$	4·0 gm.
$MgCl_2.6H_2O$	10·0 gm.
Glucose	20·0 gm.

Made up with distilled water to 2 l.

400 ml. amounts are autoclaved at 10 lb pressure for 20 min. in screw-capped bottles. When required, the contents of one bottle are diluted with distilled water to give a total of 4 l. of modified glucosol. This is dispensed in 250 ml. amounts and again autoclaved.

(ii) Sodium phosphate (ten times concentrated solution): 94·73 gm. of Na_2HPO_4 are made up to 2 l. with distilled water, and distributed in five bottles of 400 ml. each, which are stored at 4° C.

(iii) Potassium phosphate (ten times concentrated solution): 90·78 gm. of KH_2PO_4 are made up to 2 l. with distilled water, and distributed in five bottles of 400 ml. each, which are stored at 4° C.

(iv) Phosphate buffer (pH 6·8):

Sodium phosphate concentrate ((ii) above)	400 ml.
Potassium phosphate concentrate ((iii) above)	400 ml.
Distilled water	3200 ml.

250 ml. amounts are autoclaved at 10 lb. for 20 min. Sometimes a slight precipitate develops after autoclaving, but this does not seem to affect the results.

(*b*) M/100 phosphate-buffered saline (pH 6·8): 400 ml. phosphate buffer (pH 6·8) + 2267 ml. 0·85 % chloride solution. Approximately 400 ml. amounts in screw-capped bottles are autoclaved at 10 lb. for 20 min.

(*c*) Concentrated antibiotics solution:

(i) 1 gm. streptomycin base is dissolved in 5 ml. M/100 phosphate-buffered saline, and distributed in 1 ml. amounts in screw-capped bottles. These are stored at 4° C. for not longer than one month before use.

(ii) 1 mega unit of crystalline penicillin G is dissolved in 19 ml. M/100 phosphate-buffered saline, and added to 1 ml. of the stock streptomycin concentrate.

The final concentrated antibiotics solution contains 10,000 μg. of streptomycin and 50,000 units of penicillin per ml. It is used after storage at 4° C. for not more than 7 days.

(*d*) 10 % horse serum-saline:

50 ml. of sterile (filtered) horse serum + 450 ml. 0·85 % saline. This is dispensed with a sterile automatic pipetting syringe in 4·5 ml. aliquots into 'bijou' (6 ml.) screw-capped bottles, and frozen at −15° C. until required.

Ten-fold and four-fold virus dilutions are made by adding respectively 0·5 ml. or 1·5 ml. of virus suspension to 4·5 ml. of serum saline, and mixing by inversion ten times.

(2) *Glassware*

A test tube ($5 \times \frac{1}{2}$ in.) is used to hold one membrane piece and 1·5 ml. of buffered modified glucosol. The tubes are initially cleaned in a sulphuric acid-potassium dichromate bath and thoroughly rinsed. Thereafter, they are merely washed out with tap water and drained dry between tests. The tubes are not closed individually with any form of stopper during titrations.

(3) *Baskets*

Ordinary laboratory wire mesh baskets, $6 \times 6 \times 6$ in., are labelled on their fronts with identifying numerals. Each holds 100 tubes in 10 rows of 10, separated by partitions 5×4 in. high, cut from tin plate. Between the third and fourth rows, fifth and sixth rows and eighth and ninth rows, (numbering from the back), there are higher partitions, with tops painted distinctively to aid quick identification of the rows. A loose-fitting flanged tin plate lid rests on and covers the mouth of the tubes. The baskets with lids in position are sterilized in the hot air oven at 160° C. for 1 hr. before use.

(4) *Shaking machine*

A $\frac{1}{4}$ h.p. electric motor is connected through a belt and pulley reduction drive and an eccentric arm to a trolley. This was built from 'Meccano' and runs horizontally on ten flanged wheels resting on guide rails. It is pulled backwards and forwards 105 times per minute through a horizontal distance of 1·8 in. Mounted on it is a 20 in.-long platform inclined at 15° to the horizontal. The baskets are placed on this platform fronts uppermost, so that the long axes of the tubes are parallel with it. The tilt of the platform was chosen so that, during each to-and-fro cycle of the machine, there was as great a flow of the fluid up and down the tubes as could be obtained without any danger of spilling. Probably any robust laboratory shaker could be used if a suitable inclined platform is mounted on it.

(5) *Virus strains*

The D.S.P. strain of influenza A virus (Hoyle, 1948) was used principally, but experiments with a PR 8 strain of influenza A and a Lee strain of influenza B are also described.* Many titrations were carried out with samples of virus diluted 1/10 in horse serum (previously heated at 56° C./$\frac{1}{2}$ hr.), and after rapid freezing stored in hard glass ampoules at −70° C. A new ampoule was used for each titration.

(6) *Eggs*

Fertile eggs from a single pedigree flock of White Leghorns were used throughout. They were incubated for 13 days before use, unless otherwise stated.

* We are indebted to Dr W. Henle of the Children's Hospital, Philadelphia, U.S.A., for providing these strains.

(7) *Infectivity titrations in eggs, haemagglutinin titrations, etc.*

These and other techniques employed have been described recently (Finter & Beale, 1956; Beale & Finter, 1956).

PROCEDURE

Preparation of membrane pieces and inoculation of virus

The test will be described as it has usually been carried out, with 600 pieces of chorio-allantoic membrane cut from twenty thirteenth-day eggs.

Sterile equipment and chilled sterile solutions are used. 500 ml. of modified glucosol and 500 ml. of phosphate buffer (pH 6·8) are mixed, and 10 ml. of the concentrated antibiotics solution are added. This gives 1 l. of buffered modified glucosol, with 500 units of penicillin and 100 μg. of streptomycin per ml. With a sterile automatic pipetting syringe 1·5 ml. is added to every tube in six baskets.

The entire surface of the shell of each egg is wiped lightly with a cotton wool swab, which has been soaked in 70 % alcohol and squeezed nearly dry. A disk of shell about 1 in. in diameter is cut away at the pointed end with sterile scissors, and the contents of the egg are carefully decanted, leaving the 'parietal' chorio-allantoic membrane still lining the inside of the shell. After draining on a sterile egg card, the membrane is washed once *in situ* by filling the shell with chilled M/100 phosphate-buffered saline and decanting. Then starting from the cut edge, a strip of shell 7–8 mm. in width, and with the chorio-allantoic membrane still attached, is cut spirally from the egg. From time to time this strip is cut into lengths of about 25 mm., and the rectangles of shell and membrane (*membrane pieces*) fall into a Petri dish containing 20–30 ml. of buffered modified glucosol. Between twenty-five and forty pieces are obtained from an egg, with an average of over thirty. All pieces from one egg are put into one Petri dish, and the dishes are numbered from 1 to 20 corresponding to the twenty eggs. The membrane pieces are added to the tubes in a systematic manner. Starting with Petri dish no. 1, one membrane piece is put into each of the ten tubes in the first vertical column of baskets I, II and III, a total of thirty tubes (see Table 1). Similarly, one membrane piece from Petri dish no. 2 is added to each tube in the second column in these three baskets, and so on until those from dish no. 10 go into the tenth and final column. Thus in each horizontal row, the first tube contains a membrane piece taken from the first Petri dish (i.e. egg 1), the second tube a piece from egg 2, the third from egg 3, etc., and all thirty rows in these three baskets contain replicate sets of membrane pieces. The membrane pieces from eggs 11–20 are similarly dispensed into baskets IV, V and VI.

The membrane pieces are transferred from the Petri dishes to the mouths of the tubes with fine pointed forceps, only the edge of the piece being grasped. From time to time each basket is gently rapped against the bench, until the membrane pieces slide right down and are immersed in the 1·5 ml. of fluid at the bottom of the tubes. This is checked by examining the tubes through the wire mesh bottom of the baskets, since it has been found that after more than a brief period out of fluid, virus multiplication in membrane pieces is impaired. It has proved con-

Table 1. *Layout of experiment for testing reproducibility of titration method (see text for explanation)*

Row	Log of virus dilution	No. of egg from which all membrane pieces in vertical columns derived: 1	2	3	4	5	6	7	8	9	10	Replicate sets from eggs nos. 1–5. Positive score ($n=5$)	Titre (log MP_{50} per drop)
1	−8·7	0	0	0	+	0						1	
2	−8·1	0	0	0	0	0						0	
3	−7·5	+	+	0	0	0						2	7·44
4	−6·9	+	+	+	+	+						5	
5	−6·3	+	+	+	+	+						5	
		Basket I											
6	−8·7	0	0	0	0	0						0	
7	−8·1	0	0	0	0	0						0	
8	−7·5	+	0	0	+	+						3	7·56
9	−6·9	+	+	+	+	+						5	
10	−6·3	+	+	+	+	+						5	
1	−8·7	0	0	0	0	0						0	
2	−8·1	0	0	0	0	0						0	
3	−7·5	+	0	0	+	0						2	7·44
4	−6·9	+	+	+	+	+						5	
5	−6·3	+	+	+	+	+						5	
		Basket II											
6	−8·7	0	0	0	0	0						0	
7	−8·1	0	0	0	+	0						1	
8	−7·5	0	+	0	0	0						1	7·42
9	−6·9	+	+	+	+	+						5	
10	−6·3	+	+	+	+	+						5	
1	−8·7	0	0	0	0	0						0	
2	−8·1	0	0	0	0	0						0	
3	−7·5	0	+	+	0	0						2	7·32
4	−6·9	0	+	+	+	+						4	
5	−6·3	+	+	+	+	+						5	
		Basket III											
		Front five rows not used in this experiment.											

Dilution	Egg no. 1	2	3	4	5	
−8·7	0	0	0	1	0	Results in tests with individual eggs ($n=5$)
−8·1	0	0	0	1	0	
−7·5	3	3	1	2	1	
−6·9	4	5	5	5	5	
−6·3	5	5	5	5	5	
Titre (log MP_{50} per drop)	7·44	7·32	7·32	7·56	7·57	

The layout with baskets IV, V and VI was exactly comparable, but with pieces from eggs 11–20. + = positive haemagglutination in tube at harvest. 0 = negative haemagglutination in tube at harvest. n = number of membrane pieces used to test each virus dilution. MP_{50} = 50% membrane piece infectivity dose.

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venient to dispense membrane pieces from two Petri dishes at a time, filling adjacent vertical columns and tapping them down in each basket in turn. If less than thirty suitable membrane pieces are obtained from any egg, surplus pieces from other eggs are used to make up the deficiencies.

When every tube in a basket has received its appropriate membrane piece, the lid is flamed and replaced, and the basket put at 4° C. until the virus dilutions are added.

Dilutions of the virus preparations are made in 10% serum saline, with five four-fold steps over the appropriate range when the approximate titre of the preparation is known, but otherwise five ten-fold steps. Starting with the greatest dilution of the first virus preparation, a single drop (0·025 ml.) is added with a calibrated dropping pipette to each tube in the first horizontal row of basket I. With the same pipette, drops of the remaining dilutions are in turn added to the second, third, fourth and fifth horizontal rows of this basket. With another dropping pipette the five dilutions of the second virus preparation are similarly added to rows 6–10 of this basket. Table 1 illustrates the layout for five titrations of a single set of virus dilutions (these are discussed below in connexion with the reproducibility of the results). After the dilutions have been added, the basket lid is flamed and replaced. The basket is put on the inclined platform of the shaking machine in the 37° C. hot room, and the motor switched on. Dilutions of the next two virus preparations are made and similarly added to the next basket, and so on in turn with the remaining baskets and virus preparations. If each dilution is to be tested in twenty membrane pieces, two baskets containing pieces from eggs 1–10 and 11–20, respectively, are used, and the dilutions are added to corresponding rows in the two baskets. After 18–30 hr., the baskets are removed from the machine and stood upright so that the tubes are vertical and the membrane pieces immersed in the fluid. They are left at 37° C. for a total of 66–72 hr. after addition of the virus dilutions.

Harvesting

Each tube is removed in turn, and the fluid decanted into a similar clean tube, which is returned to the same position in the basket. When all the tubes have been harvested, one drop (0·03 ml.) of a guinea-pig red cell suspension (3 ml. of washed and packed red cells + 21 ml. of saline) is added to every tube. The baskets are shaken vigorously and then left undisturbed for $1\frac{1}{2}$ hr. at room temperature on a plate glass platform. The tubes are now transilluminated from above, and the number in each row with positive patterns of haemagglutination are observed in an inclined mirror placed under the baskets. Tubes with difficult or doubtful patterns of agglutination are removed and examined individually, and if there is still doubt (as occurs with less than 1% of tubes) are counted as negative. From the percentage of tubes with positive patterns of haemagglutination after each dilution of virus, an estimate of the infectivity titre in 50% membrane piece infectivity doses (MP_{50}) per drop (0·025 ml) is calculated by the three-span moving average method of Thompson (1947), most conveniently using the tables of Weil (1952). For part of the analysis of the present data, a simplified form of probit analysis (Berkson, 1955) has been used; the MP_{50} estimates by the two methods agree closely. Results are usually expressed as MP_{50} per ml.

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Washing up

(*a*) Tubes containing membrane pieces (left behind when fluids decanted at harvest): the membrane pieces are shaken out, and the tubes rinsed three times in tap water and drained dry. After being heated in the hot air oven to destroy any remaining virus, they are used to receive the fluids decanted when the next experiment is harvested.

(*b*) Tubes containing the decanted fluid and red blood cells: these are washed out in the baskets with a jet of water from a glass tube, which is pushed down to the bottom of each tube in turn. After draining dry while inverted, the baskets are sterilized in the hot air oven with their lids in position, ready for use in the next set of titrations.

Time required to perform titrations

About 1 hr. has generally been required for two workers to set up a test with six baskets of 100 tubes, each containing a membrane piece and glucosol.

With the 600 membrane pieces, obtained from 20 eggs, a number of virus preparations ranging from 24 (with $n = 5$*) to 3 (with $n = 40$) can be tested in parallel, depending on the reliability desired for the results.

It has also generally taken one hour for two workers to harvest such a test, add cells, read results, and wash up all glass-ware concerned.

Comments on the final technique adopted

(1) *Bacterial contamination*

The egg shells are handled and cut up with freshly washed, but ungloved hands, and the Petri dishes and tubes containing the membrane pieces are exposed to the air for varying periods of time. Nevertheless, trouble has not been encountered from growth of bacteria, presumably because of the large amounts of penicillin and streptomycin in each tube. Subcultures from the tubes on to blood-agar at the time of harvest have yielded no growth of bacteria. The amounts of antibiotic drugs used appear not to inhibit virus multiplication. In an experiment the same final concentrations of haemagglutinins were found in tubes containing the usual amounts of penicillin and streptomycin, and in parallel tubes with twenty-five times smaller amounts, and no bacterial growth was observed in blood-agar subcultures from either set of tubes. Slight growth of moulds occurs usually in about 5 % of tubes, but without seeming to interfere with virus multiplication or the formation of haemagglutinins.

(2) *Age of eggs*

Eggs which have been incubated for 13 days are most suitable for titrations of the D.S.P. and PR 8 strains of influenza A virus. When six preparations of D.S.P. virus were titrated in parallel in membrane pieces cut from thirteenth- and

* n = number of membrane pieces used to test each virus dilution. Data on the minimum significant differences between 50 % end-points are given in Table 3.

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fifteenth-day eggs ($n = 10$, $d = 0{\cdot}6$* for each), the titres were all lower with the fifteenth-day eggs, the mean difference being 0·49 log units. In a similar experiment, there was no significant difference between the results with membrane pieces from twelfth- and thirteenth-day eggs. Moreover, the chorio-allantoic membrane of a twelfth-day egg is liable to separate from the shell when membrane pieces are cut. However, with the Lee strain of influenza B virus, much more consistent results, and titres some seven to ten times higher, have been obtained with membrane pieces from twelfth-day eggs as compared with from thirteenth-day eggs.

(3) *Size of the membrane pieces*

Fulton & Armitage (1951) suggest that in tests of this type, the actual size of the membrane piece is not critical, provided it is big enough (approximately 150 mm.^{2}). With the present technique rectangular pieces of a fairly consistent size can be cut. The mean dimensions of twenty pieces chosen at random from a batch of 600 were $25{\cdot}2 \times 7{\cdot}0$ mm. (mean area = 177 mm.^{2}, standard deviation = 20·8 mm.^{2}). Wider pieces will not fit in the tubes used. Assuming that the cells of the entodermal layer of the allantois are 10 μ in diameter (Henle, 1950), a membrane piece will on the average contain about $10^{6{\cdot}35}$ cells.

(4) *Selection of membrane pieces*

The Lee strain of influenza B apparently multiplies equally in allantoic and chorionic cells (Tamm & Tyrrell, 1954). In contrast, influenza A virus adsorbed to cells of the chorion only gives rise to small amounts of further infective virus (Fulton & Isaacs, 1953). Therefore, when membrane pieces are cut, if the chorio-allantoic membrane separates extensively from the underlying shell membrane of any one, with consequent exposure of its chorionic surface, this is not used for titration. But when dilutions of a D.S.P. virus preparation were titrated using pieces of chorio-allantoic membrane which had been deliberately detached from membrane pieces, the titre obtained was only 0·5 log lower (with $n = 20$; $d = 0{\cdot}6$) than when the same dilutions were titrated with membrane pieces as usual.

(5) *Agitation of the tubes*

Fairly vigorous agitation is necessary for at least a short time after adding virus to the tubes, or irregular results and lower titres are obtained. The adequacy of the shaking in the standard technique was checked in the following experiment. Five dilutions in four-fold steps of a standard virus preparation were each added to two rows of tubes in six baskets. Two baskets were placed on the platform of the shaker as usual, with their tubes inclined at 15° to the horizontal. Another two baskets were mounted with the tubes inclined at only 5°, so that at each to-and-fro cycle of the platform, the fluid ran up and down them considerably more than usual. The titres from the two pairs of baskets were respectively $10^{8{\cdot}85}$ and $10^{8{\cdot}89}$ MP_{50}/ml. ($n = 40$; $d = 0{\cdot}6$). Thus increased agitation led to no apparent in-

* d = the log dilution step.

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crease in titre. The remaining two baskets were placed on the machine with their tubes sloped at approximately 25° to the horizontal. There was consequently less agitation than usual, and a slightly but significantly lower titre of $10^{8\cdot56}$ MP_{50}/ml. was obtained.

Shaking baskets for more than 18–24 hr. did not improve results, and shaking for only 6 hr. appeared to be sufficient. In an experiment, titres of $10^{8\cdot65}$ and $10^{8\cdot56}$ were obtained from replicate baskets shaken for 6 and 23 hr., respectively ($n=40$; $d=0\cdot6$). But much lower titres were obtained when baskets were not shaken at all after adding virus, or were merely shaken vigorously by hand for a short time.

(6) *Glassware*

In Fulton's technique (Fulton & Armitage, 1951), specially designed perspex trays with drilled depressions were used to hold the pieces of chorio-allantoic membrane. In the present study glass tubes have been preferred for this purpose, since they are readily available, and can be sterilized by heat. Further, they permit more vigorous agitation to be given without risk of spilling, though this occasionally happened in early experiments in which shorter tubes (3 in. × ½ in.) were used. In addition, glass tubes have been used to receive the fluids decanted in the harvest. With the simple washing up procedure employed, the use of glass tubes has involved little additional labour, and has proved more convenient than the use of available plastic trays.

(7) *Virus dilutions*

In early experiments the membrane pieces were put into empty tubes, and 1·5 ml. amounts of virus diluted in buffered modified glucosol were added appropriately. Rather irregular results and comparatively low titres were obtained, possibly because some drying of the membrane pieces may have occurred before all the dilutions could be added. In addition, glucosol, which contains no protein, is probably inferior to serum-saline as a diluent for virus preparations.

(8) *Variation in the inoculum size*

Radio-active methionine was added to a sample of the 10 % horse serum-saline diluent. A single drop of the mixture was delivered with a standard dropping pipette on to each of ten metal dishes. After drying, the radioactivity of each was measured with a Geiger counter. From the counts of the individual drops, the coefficient of variation of the drop volume was found to be about 5 %. Consideration of equation (10) of Armitage & Spicer (1956) shows that a variation of this order of magnitude in the inoculum volume will have a negligible effect on the slope of the dose-response curve.

(9) *Time of harvest*

Harvests were usually made 66–72 hr. after adding virus to the tubes. With D.S.P. virus, incubation for longer periods (96 or 120 hr.) did not lead to increased titres, and 48 hr. of incubation was probably sufficient. In replicate titrations

of the same preparation with $n = 20$ and $d = 0{\cdot}6$, the titres obtained from harvests at 48 hr. and at 72 hr. were $10^{8{\cdot}66}$ and $10^{8{\cdot}75}$ MP_{50}/ml., respectively, which do not differ significantly. With the Lee strain of influenza B virus, incubation for 96 hr. was necessary.

(10) *Technique of harvesting*

An alternative method is to remove the membrane pieces from the tubes with forceps, which are plunged into boiling water and cooled in chilled saline between each tube. The red blood cell suspension can then be added to the original tubes to test for haemagglutinins. However, with $5 \times \frac{1}{2}$ in. tubes, this takes longer than decanting the fluids and washing up both lots of tubes.

(11) *Production of haemagglutinins in membrane pieces*

After intra-allantoic inoculation of either a large or small amount of D.S.P. virus into a thirteenth-day egg, the allantoic fluid will finally contain approximately the same total amount of haemagglutinins (about $10^{4{\cdot}3}$ haemagglutinin (HA) units). The time at which the final amount is reached will, however, depend on the inoculum size. After a large inoculum all susceptible cells in the allantoic sac will be infected, and thus contribute to the total haemagglutinin production, more quickly than after a small one. In terms of surface area, the allantoic cells of a membrane piece are probably equivalent to about one-fortieth of those of the whole allantoic sac. Thus a membrane piece might be expected to produce one fortieth of $10^{4{\cdot}3}$ HA units, i.e. $10^{2{\cdot}7}$ HA units. But even 72 hr. after a large inoculum of virus, the 1·5 ml. of fluid in a titration tube usually contains a total of only $10^{2{\cdot}2}$ HA units, and after a small inoculum, when several cycles of multiplication must occur before all susceptible cells are infected, the final amount may be only $10^{1{\cdot}7}$ HA units. This suggests that there is a progressive deterioration with time in the capacity of membrane pieces to form haemagglutinins, as would be expected from the histological studies of Fulton (Fulton & Armitage, 1951). Membrane pieces may still form haemagglutinins until between 30 and 40 hr. after adding virus, as shown by increasing titres in the fluids, but the rate appears to be less than in the intact egg. Possibly some of the haemagglutinins formed are adsorbed to the external surface of the shell.

RESULTS

(A) *Results with the D.S.P. strain of influenza A virus*

Reproducibility within an experiment: results of titrations with membrane pieces from individual eggs and from groups of eggs.

Six baskets were filled with membrane pieces from twenty eggs in the sequence described. Five dilutions of a standard allantoic fluid preparation were made in four-fold steps from $10^{-6{\cdot}3}$ to $10^{-8{\cdot}7}$. A single drop of dilution $10^{-8{\cdot}7}$ was added to each tube in the first horizontal row of all six baskets, and also to each tube in the sixth row of baskets I, II, IV and V (see Table 1). With the same dropping pipette, the other dilutions were added in turn to the succeeding rows, until finally the

dilution $10^{-6\cdot3}$ was added to the fifth row of all the baskets, and also to the tenth row of baskets I, II, IV and V. When the test was harvested, the result in each tube, i.e. positive or negative haemagglutination, was recorded.

In the horizontal rows of tubes, the same virus dilutions were tested five times with replicate sets of five membrane pieces derived from eggs nos. 1–5, i.e. twice in basket I, twice in basket II, and once in basket III. The scores of positive tubes in these five titrations are shown on the right of Table 1 together with their titres in log MP_{50} as estimated by Thompson's method. There were similarly five titrations with replicate sets of five membrane pieces at each dilution derived from eggs 6–10; five from eggs 11–15; and five from eggs 16–20. The 50 % end-points of all these twenty titrations are shown in Table 2*A*.

Table 2. *Results of titrations of a single set of virus dilutions to provide data on reproducibility*

(*A*) Results with replicate sets of five membrane pieces (one piece in each set from each of the same five eggs) at each of five virus dilutions.

Eggs nos: 1–5	6–10	11–15	16–20
	Titre (log MP_{50} per drop)		
7·44	7·12	7·56	7·42
7·56	7·72	7·42	7·44
7·44	7·40	7·20	7·68
7·42	7·26	7·32	7·40
7·32	7·80	7·57	7·42

(*B*) Results with five membrane pieces all from the same egg at each of five virus dilutions.

Egg no.	Titre (log MP_{50} per drop)	Egg no.	Titre (log MP_{50} per drop)	Egg no.	Titre (log MP_{50} per drop)	Egg no.	Titre (log MP_{50} per drop)
1	7·44	6	7·00	11	7·12	16	7·44
2	7·56	7	7·72	12	7·56	17	7·40
3	7·32	8	7·42	13	7·44	18	7·40
4	7·57	9	7·56	14	7·68	19	7·40
5	7·32	10	7·80	15	7·32	20	7·68

In the vertical columns of tubes, each virus dilution was tested with five membrane pieces derived from a single egg. The score of positive tubes for the titration carried out entirely with membrane pieces from egg 1 is shown at the bottom of Table 1, and similarly for the titrations with pieces all from egg 2, all from egg 3, etc. The 50 % end-points of the twenty titrations in individual eggs are shown in Table 2*B*.

An analysis of variance of the results in Table 2*A*, and the corresponding results in a second and exactly comparable experiment, provided no evidence that the MP_{50} end-point varied systematically either from one group of five eggs to another (e.g. with pieces from eggs 1–5 versus those from eggs 6–10), or between replicate titrations with a single group of five eggs (e.g. the five titrations with replicate sets of pieces from eggs 1–5). This was confirmed by a fuller analysis of the same data, in which the results for individual eggs were treated separately. From the two

reproducibility experiments, the standard error of the 50% end-point of a titration with $n=5$, $d=0{\cdot}6$, is estimated (on 38 degrees of freedom) as 0·215 log units. This standard error applies if the end-point is estimated by Thompson's method, and would be slightly reduced by a more efficient method such as probit analysis; Thompson's approximate formula for the standard error gives a pooled value of 0·24, which agrees satisfactorily with the observed value of 0·215.

The statistical analysis suggests that membrane pieces from different eggs may be taken as equivalent in infectivity tests. There would appear, therefore, to be no need to allocate them to the tubes systematically. Nevertheless, the procedure described is convenient, and clearly will diminish the chance of a very low (or high) titre due to unusually low (or high) sensitivity of pieces from one particular egg, though no such extreme was noted among the forty eggs in the above two experiments. On the other hand, in experiments with the Lee strain of influenza B virus which are discussed below, there was appreciable variation in the susceptibility of pieces from different eggs.

Table 3. *Titrations of the D.S.P. strain of influenza A virus. Statistical data on the reliability of a* 50% *titration result (expressed in log* MP_{50}*/ml., as estimated by Thompson's method), and on the minimum significant difference between two end-points, for different values of n and d*

	log dilution step					
	$d=0{\cdot}6$				$d=1{\cdot}0$	
No. of membrane pieces used to test each virus dilution	$n=5$	$n=10$	$n=20$	$n=40$	$n=10$	$n=20$
Standard error of end-point (σ)	0·21	0·15	0·11	0·08	0·20	0·14
Range, on either side of the *true* end-point, within which a single estimate will lie						
(*a*) in 95 tests out of 100 ($1{\cdot}96\sigma$)	0·42	0·30	0·21	0·15	0·38	0·27
(*b*) in 99 tests out of 100 ($2{\cdot}58\sigma$)	0·55	0·39	0·28	0·20	0·51	0·36
Minimum difference between end-points of two preparations which is significant						
(*a*) at the 5% level ($1{\cdot}96\sqrt{2}\sigma$)	0·60	0·42	0·30	0·21	0·54	0·39
(*b*) at the 1% level ($2{\cdot}58\sqrt{2}\sigma$)	0·78	0·55	0·39	0·28	0·72	0·51

Table 3 shows the standard error of the end-point (σ), and various associated quantities, for $n=5$, 10, 20 and 40 with $d=0{\cdot}6$, and also for $n=10$ and 20, with $d=1{\cdot}0$. The standard error of 0·215 given above corresponds to $n=5$, $d=0{\cdot}6$, and the other values for σ are obtained from the formula $0{\cdot}215\sqrt{\{(5/n)\,(d/0{\cdot}6)\}}$. With $d=0{\cdot}6$, a four-fold (0·6 log units) or greater difference in the end-point of two D.S.P. virus preparations will be significant at the 5% level when $n=5$, and a two-fold (0·3 log units) difference when $n=20$.

As mentioned below, the slope of the dose-response curves, and hence the standard errors of the end-points, varied slightly from one occasion to another. The data of Table 3 should thus be considered as indicative of the approximate variability of the results with the method rather than as absolute values. Furthermore, the

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Table 4. *Log 50 % infectivity titres (log MP_{50} per ml.) obtained for the same virus preparations on a number of different occasions*

	Virus preparation tested												
	Source (AF or CAM)	Nature	EID_{50}/ HA log	27 July	10 Aug.	16 Aug.	23 Aug.	30 Aug.	6 Sept.	13 Sept.	27 Sept.	11 Oct.	31 Oct.	Weighted mean ± S.E.
(A)	AF	ST	6·10	—	—	—	—	—	8·82	8·91*	9·08*	—	8·95*	8·98 ± 0·03
(B)	AF	ST	5·85	7·96†	8·15	8·37	8·47	—	—	—	—	—	—	8·28 ± 0·06
(C)	AF	IC	3·95	6·03†	6·31	6·58	—	—	—	—	—	6·36‡	—	6·36 ± 0·05
(D)	AF	ST	5·30	7·82†	7·80	7·98	7·79	—	—	—	—	—	—	7·85 ± 0·06
(E)	AF	Inact.	4·60	—	—	—	7·10	7·05	6·87	—	—	6·99	—	7·01 ± 0·05
(F)	AF	Inact.	3·90	—	5·96	5·94	6·01	5·94	—	—	—	—	—	5·96 ± 0·05
(G)	CAM	ST	5·85	—	—	—	8·54	8·57	8·52	—	8·65	—	8·57*	8·57 ± 0·04
(H)	CAM	IC	3·90	—	—	5·92§	—	5·93	5·90	6·04	—	—	—	5·96 ± 0·06
(J)	CAM	IC	3·60	—	(4·95)§	—	—	5·68	5·54	—	—	5·67‡	—	5·64 ± 0·06

Standard error of logarithmic titres (obtained by separate probit analysis for each experiment) lie between 0·10 and 0·13 unless otherwise stated.

AF = allantoic fluid. CAM = chorio-allantoic membrane extract. ST = standard virus. IC = incomplete virus. Inact. = inactivated allantoic fluid virus (heated 37° C. *in vitro*).

* Standard error = 0·05. † Standard error = 0·18–0·19. ‡ Standard error = 0·08. § Standard error = 0·14–0·16. ( ) Value omitted from weighted mean.

data would not necessarily apply to comparable titrations by this technique performed by other workers and with other strains of influenza virus, though results of the same order could reasonably be expected. Similar comments, of course, apply to data on the reproducibility of titrations in eggs.

Reproducibility of results on different occasions

Nine preparations of D.S.P. virus were titrated each on at least four occasions during a period of 3 months. They included standard and incomplete allantoic fluid virus, allantoic fluid virus which had been partially inactivated by heating at 37° C., and standard and incomplete chorio-allantoic membrane virus. Standard virus was harvested from eggs 18–24 hr. after inoculation of highly diluted seed, and the EID_{50}/HA ratio (i.e. the ratio of the 50 % end-point in whole eggs to the haemagglutinin titre) was usually about $10^{6.5}$ for allantoic fluid virus, and slightly less for chorio-allantoic membrane virus. Incomplete virus was prepared by the technique of von Magnus (1951*b*) and had lower values for the EID_{50}/HA ratio. Results of all titrations of the nine preparations are shown in Table 4. The log MP_{50} values were estimated by a simplified form of probit analysis (Berkson, 1955). In the different titrations the value of d was 0·6 throughout, but n varied from 10 to 100, and the standard errors of the log MP_{50} estimates correspondingly from 0·19 to 0·05. There was no evidence of differences between the slopes of the probit lines of different preparations titrated in parallel on the same day (shown in the same vertical columns of Table 4). Therefore, parallel probit lines were fitted for all such preparations. But the slope was determined separately for each set of titrations, since it varied significantly from one occasion to another, the lowest and highest values observed being $1{\cdot}27 \pm 0{\cdot}10$ and $1{\cdot}97 \pm 0{\cdot}14$. It will be seen from Table 4 that, with one exception, the titres obtained on different occasions for each virus preparation did not differ significantly. The exception was the first titration of preparation (J); this was possibly the result of some dilution error, and was not taken into account in the subsequent calculations.

Further evidence on reproducibility is provided by the following estimates of log MP_{50} (calculated by Thompson's method) from nine titrations of preparation (A), all with $n = 20$, $d = 0{\cdot}6$, performed during a period of 8 months: 8·73, 8·84, 9·00, 8·93, 8·78, 8·97, 8·80, 8·75 and 8·83. These values have a standard deviation of 0·10 (compare that of 0·11 from Table 3 for replicate titrations in a single experiment with the same values for n and d).

Relation of egg infectivity titres (EID_{50}) to membrane piece infectivity titres (MP_{50})

Seventeen D.S.P. virus preparations were titrated with membrane pieces on two or more occasions, with $n = 20$, $d = 0{\cdot}6$. The preparations included the nine shown in Table 4, and their EID_{50}/HA ratios ranged from $10^{6.4}$ to $10^{4.6}$. For estimation of the titre, a separate slope was fitted for each set of membrane piece titrations, as described earlier. The separate estimates of log MP_{50} obtained for each preparation were consistent (with the single exception noted above, which was ignored), and a pooled value x_{MP} was obtained, together with its standard error.

Each preparation was also titrated at least twice in eggs. Three or four virus dilutions were inoculated into groups of five or six fertile eggs, with $d = 1{\cdot}0$ or $0{\cdot}6$ over the anticipated range in the first titration, and $d = 0{\cdot}6$ over the indicated range in the second. On analysis, no significant differences between the slopes on different occasions were found, perhaps because the sampling errors were much larger than for the membrane piece titrations. The pooled slope was $1{\cdot}12 \pm 0{\cdot}06$ and this was used to estimate the log EID_{50} end-point of each titration. A pooled value, x_E, was obtained for each preparation. The sampling errors in x_E were greater than those of x_{MP}, partly because of the smaller numbers of eggs used and the larger dilution steps in some of the titrations, and partly because of the lower slope.

The log ratio $r = x_E - x_{MP}$ was calculated, with its standard error, for each preparation. Although D.S.P. virus preparations of a variety of types were tested, no significant differences in the value of r were observed between them. Mean values of r were as follows:

	No. of preparations tested	$r = x_E - x_{MP}$ mean value $\pm$ standard error
Standard allantoic fluid	4	$0{\cdot}75 \pm 0{\cdot}09$
Standard chorio-allantoic membrane	2	$0{\cdot}91 \pm 0{\cdot}15$
Incomplete allantoic fluid	3	$1{\cdot}04 \pm 0{\cdot}13$
Incomplete chorio-allantoic membrane	6	$0{\cdot}83 \pm 0{\cdot}09$
Allantoic fluid heated at 37° C.	2	$1{\cdot}08 \pm 0{\cdot}14$

There are no significant differences between these means. The overall mean value of r is $0{\cdot}88 \pm 0{\cdot}05$, i.e. taking limits of two standard errors, the titre in eggs is about 6–10 times greater than that with membrane pieces. Fig. 1 shows the relation between x_E and x_{MP} for each preparation, with the straight line representing the average relationship: $x_E = x_{MP} + 0{\cdot}88$. Titres obtained by the membrane piece technique may thus be converted to 'equivalent 50% egg infectivity doses', to provide numerical results comparable with those of egg titrations.

Dose-response curves with different virus preparations

The percentage of positive tubes recorded at each dilution tested of four D.S.P. virus preparations (A), (E), (J), and (K) are shown in Table 5. With each preparation all available results from several different experiments (with the exception of the aberrant result with (J) already mentioned) have been combined, since in these experiments there were no appreciable differences between the responses at a given dilution. (A) was the standard allantoic fluid virus used for the reproducibility experiments. Its EID_{50}/HA ratio was $10^{6{\cdot}1}$. (E) was a sample of allantoic fluid virus which had been heated at 37° C. *in vitro* for 23 hr. (EID_{50}/HA ratio = $10^{4{\cdot}6}$), while (K) was another sample heated for 65 hr. (EID_{50}/HA = $10^{2{\cdot}9}$). (J) was a chorio-allantoic membrane extract containing incomplete virus (EID_{50}/HA = $10^{3{\cdot}6}$). The estimates of MP_{50} from the individual titrations of (A), (E) and (J) are included in Table 4.

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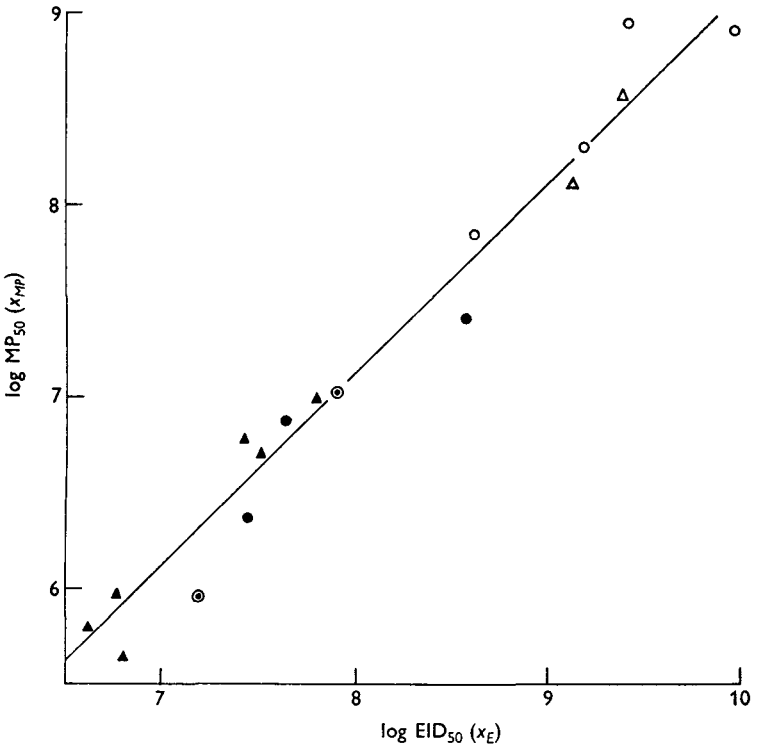

Fig. 1. Pooled membrane piece infectivity titres (x_{MP}) of seventeen D.S.P. influenza virus preparations plotted against the corresponding pooled egg infectivity titres (x_E). ○ = standard allantoic fluid virus. △ = standard chorio-allantoic membrane virus. ● = incomplete allantoic fluid virus. ▲ = incomplete chorio-allantoic membrane virus. ⊙ = allantoic fluid virus heated *in vitro* at 37° C.

Table 5. *Total numbers and proportions of positive tubes at each dilution from several titrations of four preparations of D.S.P. virus*

Preparation:	A		E		J		K	
EID_{50}/HA ratio:	$10^{6.1}$		$10^{4.6}$		$10^{3.6}$		$10^{2.9}$	
	Positive tubes			Positive tubes		Positive tubes		Positive tubes
log dilution	no.	%	log dilution	no.	%	log dilution	no.	%	log dilution	no.	%
−8·7	3/100	*3*	−7·0	0/20	*0*	−5·1	0/20	*0*	−3·7	7/40	*18*
−8·4	13/266	*5*	−6·7	1/40	*2*	−4·8	4/40	*10*	−3·1	23/40	*58*
−8·1	25/150	*17*	−6·4	3/40	*8*	−4·5	7/40	*18*	−2·8	15/19	*79*
−7·8	58/267	*22*	−6·1	5/40	*12*	−4·2	14/40	*35*	−2·5	49/60	*82*
−7·5	62/150	*41*	−5·8	10/40	*25*	−3·9	19/40	*48*	−2·2	17/20	*85*
−7·2	129/266	*48*	−5·5	15/40	*38*	−3·6	34/40	*85*	−1·9	39/60	*65*
−6·9	127/150	*85*	−5·2	24/40	*60*	−3·3	37/40	*92*	−1·6	4/20	*20*
−6·6	228/265	*86*	−4·9	31/40	*78*	−3·0	39/39	*100*	−1·3	14/59	*24*
−6·3	149/150	*99*	−4·6	40/40	*100*	−2·7	39/40	*98*			
−6·0	266/267	*100*	−4·3	40/40	*100*	−2·4	56/59	*95*			
			−4·0	20/20	*100*	−2·1	48/60	*80*			
			−3·3	20/20	*100*	−1·8	21/60	*35*			
			−1·3	20/20	*100*	−1·5	9/59	*15*			

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In Fig. 2, dose-response curves have been fitted to the data for (A) and (E) by assuming a linear relationship between the probit of the percentage of positive tubes and the log dilution. There are significant departures from the fitted curve for (A), the preparation with which the number of observations was greatest, but the discrepancy does not appear from Fig. 2 to be very serious. With both (J) and (K) there was a secondary decrease in the proportion of positive tubes, when the

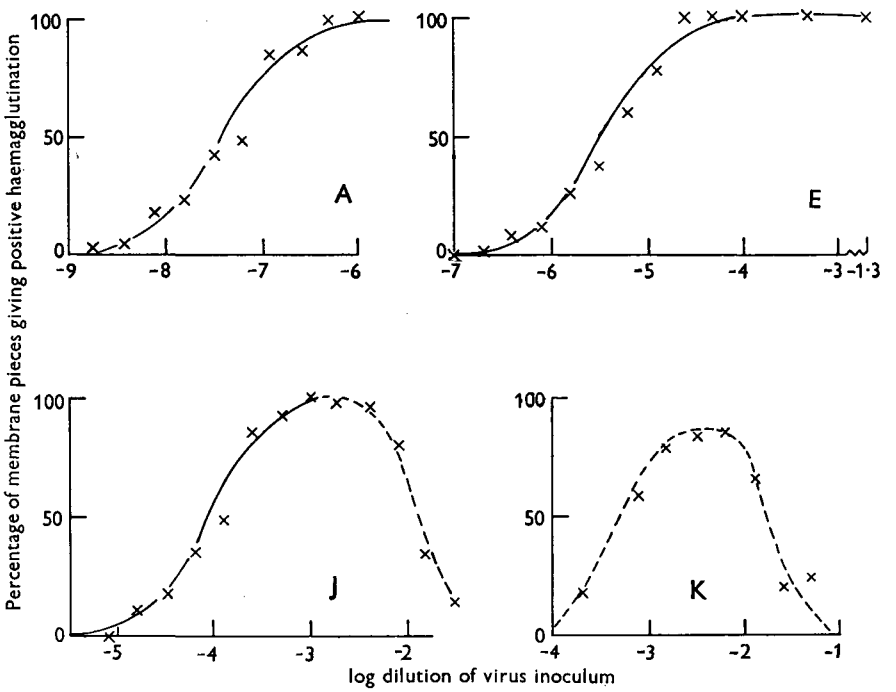

Fig. 2. Dose-response curves from membrane piece titrations of four D.S.P. virus preparations. ——, curve fitted by probit analysis; – – – –, curve fitted by eye.

concentration of virus added was increased beyond a certain level, an effect presumably due to interference. A probit curve was fitted to the results for (J) over the range indicated by a continuous line in Fig. 2, and then continued by eye for higher virus concentrations as shown by the dotted line. The data for (K) were too sparse to justify any mathematical curve-fitting. The slopes of the probit lines (with standard errors) for (A), (E) and (J) were respectively $1{\cdot}61 \pm 0{\cdot}06$, $1{\cdot}68 \pm 0{\cdot}15$ and $1{\cdot}98 \pm 0{\cdot}19$, values which do not differ significantly. In further experiments, no significant differences were found between the slopes of the probit lines of a number of other standard and incomplete virus preparations, including both allantoic fluid and chorio-allantoic membrane virus. Similar conclusions for standard and incomplete allantoic fluid virus were reached by Fazekas de St Groth (1955) from analysis of egg titration data.

Interference

In most titrations, as the concentration of virus added was increased, the percentage of tubes containing haemagglutinins increased until a 100% positive

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response was obtained; with further increase in virus, there was usually still a 100% positive response (e.g. Fig. 2(E)). But preparations containing a very high proportion of non-infective haemagglutinins sometimes gave a different result. For example, an extract of chorio-allantoic membranes harvested 6 hr. after inoculation of undiluted standard allantoic fluid contained $10^{6\cdot8}$ EID_{50}/ml., and $10^{3\cdot2}$ HA units per ml. (EID_{50}/HA ratio $= 10^{3\cdot6}$). Fig. 2(J) shows that when the concentration of virus added was greater than about $10^{-2\cdot3}$, there was a progressive decrease in the percentage of haemagglutinin-positive tubes at harvest. This was presumably due to the presence in this preparation of a relatively small number of virus particles which were not infective under the conditions of the test, and prevented multiplication of infective virus in the cells to which they were absorbed, i.e. caused interference (e.g. see Henle, 1950). In a large number of membrane piece titrations, this effect has been encountered with only four virus preparations. These all had infectivity titres in eggs between $10^{6\cdot7}$ and $10^{7\cdot2}$ EID_{50}/ml., and EID_{50}/HA ratios between $10^{3\cdot6}$ and $10^{3\cdot9}$. In titrations of a further two preparations, which had infectivity titres of $10^{6\cdot3}$ and $16^{6\cdot2}$ EID_{50}/ml. and EID_{50}/HA ratios of $10^{3\cdot6}$ and $10^{2\cdot9}$, respectively, a 100% positive haemagglutinin response was not obtained with any dilution tested. Fig. 2(K) shows the dose-response curve obtained with the second of these preparations, an allantoic virus preparation, which had been heated at 37° C. for 65 hr. A 50% membrane piece infectivity titre cannot be calculated in the usual way, though an estimate can be obtained from the graph.

(B) *Results with the* PR 8 *strain of influenza A virus*

One standard and two incomplete allantoic fluid preparations of PR 8 virus were each titrated on three occasions by the technique described. For estimation of the infectivity titres, a separate probit slope was fitted for each experiment. The overall value for the slope in all the titrations was $1\cdot21 \pm 0\cdot08$, which may be compared with values ranging from $1\cdot27 \pm 0\cdot10$ to $1\cdot97 \pm 0\cdot14$ with D.S.P. virus. The three separate estimates of log MP_{50} obtained for each preparation were consistent, and a pooled value x_{MP} was calculated.

The three PR 8 preparations were also titrated in eggs, and the pooled probit slope was calculated as $1\cdot34 \pm 0\cdot21$ (compare $1\cdot12 \pm 0\cdot06$ for D.S.P. virus in eggs). From this slope the titre in log EID_{50} per ml. was estimated for each titration, and a pooled value x_E was obtained for each preparation. The log ratio $r = x_E - x_{MP}$, and its standard error, was calculated for each of the three preparations, giving values of $1\cdot41 \pm 0\cdot20$, $1\cdot23 \pm 0\cdot20$ and $1\cdot12 \pm 0\cdot18$. These are consistent with each other, and the overall mean value of r for the PR 8 strain is $1\cdot25 \pm 0\cdot11$. This differs significantly from the value of $0\cdot88 \pm 0\cdot05$ for the D.S.P. strain. With PR 8 virus, the membrane piece technique is about 11–30 times less sensitive than infectivity titrations in eggs (as compared with the factor of 6–10 times with D.S.P. virus). The probit slopes obtained in the present series of titrations of D.S.P. and PR 8 virus, and also those calculated from egg titration data of three other authors, are shown in Table 6.

Table 6. *Slopes of lines relating probits of percentage infected to log dilution, for titrations in eggs and in membrane pieces (MP) with different influenza A virus strains.*

Author	Virus strain	Host	Slope of probit line
This paper	D.S.P.	MP	$1{\cdot}27 \pm 0{\cdot}10$ to $1{\cdot}97 \pm 0{\cdot}14$ (mean value, $1{\cdot}58 \pm 0{\cdot}05$)
		Egg	$1{\cdot}12 \pm 0{\cdot}06$
	PR 8	MP	$1{\cdot}21 \pm 0{\cdot}08$
		Egg	$1{\cdot}34 \pm 0{\cdot}21$
von Magnus* (1951*a*)	PR 8	Egg	1·47
Knight* (1944)	PR 8	Egg	1·34
Fulton & Armitage (1951)	BAR	Egg	$1{\cdot}42 \pm 0{\cdot}23$

* Values obtained by fitting probit lines to the data given by these authors. No standard error given, as the slope varied from one occasion to another.

(C) *Results with the Lee strain of influenza B virus*

Preliminary experiments indicate that different conditions are required for titrations of this strain. The best results have been obtained with membrane pieces cut from twelfth-day eggs, and when the baskets were rocked for 48 hr. after adding the virus dilutions, and harvested after incubation for a total of 96 hr. The longer incubation required compared with the influenza A strains tested is probably connected with the longer growth cycle of influenza B strains (Henle & Rosenberg, 1949).

MP_{50} titres have been obtained for two preparations of Lee virus with values approximately one-fiftieth of their EID_{50} titres (i.e. $r =$ approx. 1·7). With membrane pieces from thirteenth-day eggs, the titres obtained were seven to ten times lower still. The slopes of the dose-response curves were much lower than with D.S.P. or PR 8 virus. This suggests that there is a greater variation in the susceptibility of membrane pieces from different eggs to infection with Lee virus than with the influenza A virus strains, at least under the conditions of the experiments. In confirmation, a standard error of 0·46 was calculated from the MP_{50} estimates of titrations with membrane pieces derived from individual eggs with $n = 5$, $d = 0{\cdot}6$ (compare the value of 0·21 for similar titrations with D.S.P. virus). Variation in the ability of membranes from different eggs to support the multiplication of Lee virus *in vitro* has previously been reported by Tamm, Folkers & Horsfall (1953).

At present titrations of Lee virus are less satisfactory than those of either of the influenza A strains tested. At harvest, up to 5% of the tubes may show atypical red cell patterns, which cannot be interpreted with confidence as either positive or negative. This may account for the greater variation obtained in the results of titrations of the same preparation on different occasions than with either the PR 8 or D.S.P. strains.

DISCUSSION

From the slopes of the dose-response curves of virus infectivity titrations, some idea of the variation in susceptibility to infection among individual hosts (i.e. membrane pieces or eggs) can be obtained. For suppose that on any one occasion a proportion p of all the virus particles can initiate infection of a membrane piece, resulting in production of detectable amounts of haemagglutinins. If there is no variation in the susceptibility to infection of different membrane pieces, p will be the same for all of them. The percentage of membrane pieces infected will then be related exponentially to the concentration of virus added, and the probit of the percentage infected will be related approximately linearly to the log dilution, with a slope of about two (Peto, 1953). But in the experiments reported here, the observed slopes were all less than two. This suggests that p varies appreciably from one membrane piece to another (Moran, 1954; Armitage & Spicer, 1956). Similar considerations apply to the egg titrations. With the D.S.P. strain of influenza A virus, the slope for these was lower than that for membrane piece titrations. Thus the proportionate variation in p (expressed, say, by the coefficient of variation) must be greater among whole eggs than among membrane pieces. But reproducibility experiments with this strain of virus showed no evidence that the end-point for a titration with membrane pieces all from one egg differed from that obtained with pieces from any other egg. This suggests that under comparable conditions the allantoic cells of different eggs are on the average equally capable of supporting virus multiplication. The greater variation in p with titrations in whole eggs is thus probably due to other causes, such as differences in the composition of the allantoic fluid in individual eggs. With PR 8 virus, the probit slopes were not significantly different for titrations in eggs and with membrane pieces. With Lee influenza B virus there was evidence of variation in the susceptibility to infection of membrane pieces from individual eggs, and also of a marked difference in response of membrane pieces from eggs which had been incubated for 12 and 13 days.

If two or more virus particles must be adsorbed to a single allantoic cell of an egg or membrane piece before it is infected (i.e. further virus is formed, leading to infection of other cells and production of detectable haemagglutinins), the response curve should be steeper than the exponential (see Luria, 1953), unless the membrane pieces or eggs vary greatly in susceptibility. The slope of the probit line should also be greater than two. But the slopes obtained for incomplete virus preparations are not significantly steeper than those for standard virus preparations, and both are less than two. There is, therefore, no evidence that such a process occurs at all under the conditions of infectivity tests in eggs or in membrane pieces.

The allantoic cells in a membrane piece are slowly dying, and virus multiplication probably occurs for a shorter time than in the whole egg. Even after large inocula, the total production of haemagglutinins is only about a third of what might have been expected, and presumably after smaller inocula the amounts formed may not reach detectable levels. This is probably why membrane piece titrations give lower 50% infectivity titres than egg titrations. The differences

were most marked with Lee influenza B virus, with which the rate of multiplication is slower than with influenza A strains (Henle & Rosenberg, 1949). Possibly changes in the composition or pH of the glucose-salt solution, as suggested by Horváth (1954) might improve the sensitivity of membrane piece titrations, though preliminary experiments along these lines with D.S.P. virus have not led to any striking improvement.

Several methods for measurement of the infectivity of influenza virus preparations *in vitro* have now been described. The present technique has been developed from that of Fulton & Armitage (1951) which has been outlined already. In comparison, the present method is considerably quicker to perform, and is better adapted for large scale working, while no special equipment is needed. It is also possibly slightly more sensitive.

Surviving tissue suspensions have also been used as a source of susceptible cells for growth and titration of influenza virus in methods described by Gadjusek (1953), Horváth (1954) and Wunder, Brandon & Brinton (1954). The fullest details are given by Horváth (1954), and his method appears sensitive and gave reproducible results, though statistical data are not given.

Direct counts of plaque-forming infective particles of influenza virus can be obtained by the techniques described by Granoff (1955) and Ledinko (1955). Such methods will be very useful for special purposes, but at present seem rather elaborate and slow, requiring at least 5 days for their performance.

In order to use the membrane piece technique in another laboratory, it should only be necessary to determine the ratio of the 50 % infectivity titres in eggs and in membrane pieces for a single preparation of each influenza virus strain. Thereafter, results obtained with each strain and the exact technique employed can be converted to 'equivalent EID_{50}'. This is recommended since the results of 50 % infectivity titrations in eggs probably vary little from one laboratory to another. It will be seen from Table 6 that the probit slopes obtained for egg titrations of PR 8 virus in this and in two other laboratories were closely similar.

It may be noted that Knight (1944), using PR 8 virus, reported a standard deviation of the log EID_{50} of 0·132, with $n = 10$, $d = 10$. This value is considerably lower than that of 0·22, which would be expected by random sampling when the probit slope is 1·34. With a standard deviation of 0·22, it would be necessary to use about ten eggs per ten-fold dilution in titrations of two virus preparations, for four-fold differences in their 50 % end-points to be significant at the 5 % level, and forty eggs per ten-fold dilution for significant two-fold differences.

In order to estimate the end-point in a given virus titration with equal precision, it will be necessary to use K times as many membrane pieces as eggs. K is obtained from the relation $K = b^2_E / b^2_{MP}$, where b_E and b_{MP} denote the probit slopes of titrations with eggs and with membrane pieces respectively. From our data with D.S.P. virus, K is estimated at about 0·5, with 95 % confidence limits of about 0·4–0·7; with PR 8 virus, K is estimated at 1·25 (with limits of 0·7–2·5) which is significantly higher. In titrations of the BAR strain of influenza A virus by their similar technique, Fulton & Armitage (1951) estimated K as 0·67, with limits of 0·27–1·57.

The membrane piece technique could probably be used for studies on interference and with chemotherapeutic agents, like those reported by Tyrrell & Tamm (1955), and Tamm, Folkers, Shunk & Horsfall (1954), using a similar *in vitro* system.

SUMMARY

1. The membrane piece technique for *in vitro* titrations of the infectivity of influenza virus is described. Rectangles of shell, about 8×25 mm., with the chorio-allantoic membrane still attached (membrane pieces) are cut from thirteenth-day fertile eggs. One piece in a test-tube with glucose-buffered salt solution forms an individual assay unit. Five or more tubes are inoculated with each virus dilution. After incubation at 37° C. for 72 hr., with agitation for the first 24 hr. the fluid in each tube is tested for haemagglutinins. From the results at each dilution, an estimate of the 50% membrane piece (MP_{50}) infectivity titre is obtained.

2. Six hundred assay units, with pieces cut from twenty eggs, can be set up by two workers in 1 hr. and used for titration of between three and twenty-four individual virus preparations, depending on the reliability desired for the 50% end-point estimates.

3. With the D.S.P. and PR 8 strains of influenza A virus, the MP_{50} titres parallel the EID_{50} titres from egg titrations, but are eight times and twenty times lower, respectively. The MP_{50} : EID_{50} ratio is the same for various preparations of the same strain, including standard allantoic fluid and chorio-allantoic membrane virus, incomplete virus, and inactivated (heated) allantoic fluid virus. Preliminary experiments with Lee influenza B virus show that slightly different experimental conditions are required, and the MP_{50} titres are about fifty times less than the EID_{50} titres.

4. Consistent results have been obtained on titration of samples of the same virus preparation on a number of occasions over a period of several months.

5. A large number of membrane pieces can be used to test each virus dilution, and sampling variations in the MP_{50} estimates thus made quite small. Statistical data on the reliability of a 50% titration result, and on the minimum significant differences between two end-points, are given for different values of n, the number of membrane pieces used to test each virus dilution, and of d, the log dilution step.

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