SURVIVING TISSUE SUSPENSIONS FOR INFLUENZA VIRUS TITRATION

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(With Plates 12 and 13 and 2 Figures in the Text)

INTRODUCTION

Influenza virus which has been adapted to grow in the developing hen's egg will multiply very readily in the cells which line the allantoic sac and also in the epithelial cells which cover the sac. It has already been shown by Lahelle & Horsfall (1949) that the virus will multiply in the allantoic membrane of chick embryos which have been killed by chilling, and Bernkopf (1949) has devised a technique in which the embryo is tipped out of the egg and the virus cultivated in that part of the allantoic membrane which remains adherent to the shell.

The logical extension of this idea is to suspend the chorio-allantoic membrane in a suitable fluid containing the virus, which might then be expected to enter cells on both sides of the membrane and, after multiplying, to burst out and infect other cells. In fact, Weller & Enders (1948) used minced chorio-allantoic membrane in this way for virus cultivation.

The object of this paper is to describe a simple way of making such tissue suspensions for the specific purpose of virus titration and serum neutralization tests.

GENERAL DESCRIPTION OF THE TECHNIQUE

Preliminary experiments were made on the assumption that the aeration of the tissue would be an important factor. Accordingly chorio-allantoic membranes from 12-day embryos were removed and dropped into 250 ml. flasks containing 20 ml. of Tyrode, one membrane to each flask; influenza virus was added to the suspending fluid. The flasks were each provided with a ground-glass joint into which was fitted a tap assembly. In this way, various gas mixtures could be introduced. The flasks were placed on a rocking machine in an incubator maintained at 36° C., and they were rocked for 2 days; then the fluid in the flasks was titrated for haemagglutinin in the usual way.

It soon became clear that although rocking the flasks was of great importance, as good multiplication of the virus was obtained when the air or oxygen/CO_2 mixture in the flask was replaced with nitrogen. Thereafter a much simpler technique was adopted, the chorio-allantoic membranes being cut into pieces and distributed to screw-capped bottles of 5 or 20 ml. capacity, each bottle containing 1 or 2 ml. of suspending fluid.

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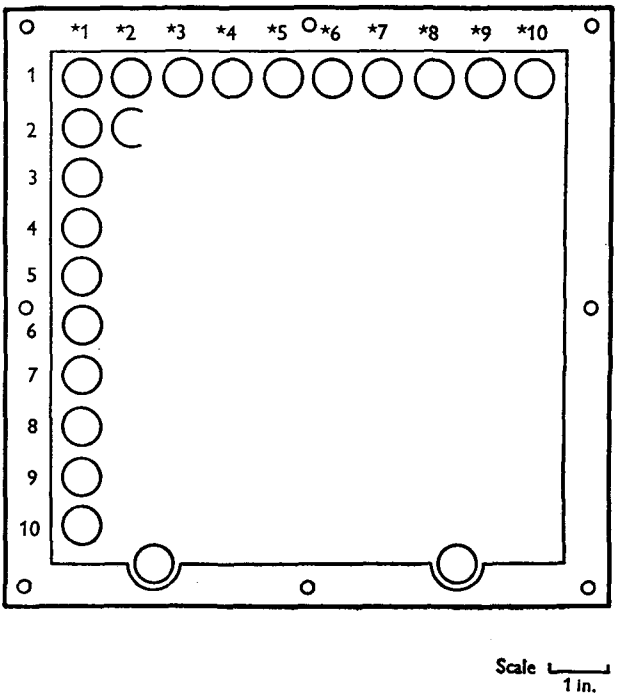
Finally, a very convenient plastic tray was devised on which it was possible to detect the presence or absence of virus in the suspending fluid without transferring the fluid to other tubes.

APPARATUS AND MATERIALS

(1) *Plastic tray* (Pl. 12 and Text-figs. 1 and 2)

The tray was made from a block of Perspex (lucite) 1 in. thick and $10\frac{3}{8}$ in. square. The block was drilled in the way which has already been described for another purpose by Salk (1948), the cups so formed being arranged in ten rows of ten. Two extra cups were provided below the tenth row to serve for controls of erythrocyte pattern in the final haemagglutination test.



Text-fig. 1. Scale drawing of plastic tray. Top view.

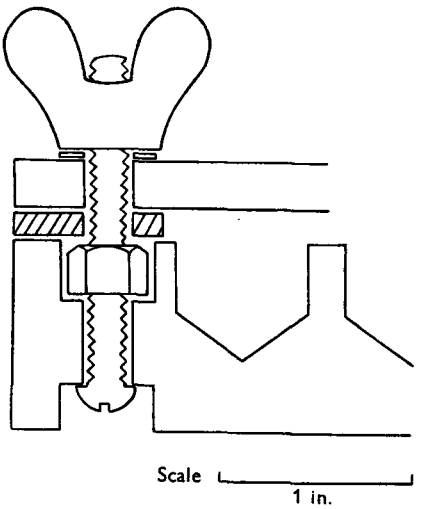
Although the exact size of the cups is clearly not of critical importance, the dimensions of those used and the lay-out of the tray are shown in Text-figs. 1 and 2.

The capacity of each cup is approximately 2 ml. At each corner of the block and at the mid-point of each side, holes were bored to contain stainless steel bolts, $1\frac{1}{2}$ in. long ($\frac{1}{4}$ B.S.F.) with locking nuts, counter-sunk. A rubber washer $\frac{1}{8}$ in. thick and $\frac{3}{4}$ in. wide was cut, and holes made so that the bolts protruded through the rubber. It is convenient to number the rows and the columns in the way indicated in Text-fig. 1 by stencilling numbers on to the rubber washer, using indian ink, and then painting over them with a thin rubber solution. A covering plate of $\frac{1}{4}$ in. Perspex was suitably drilled so that it fitted loosely over the bolts and could

be screwed down on to the rubber washer by means of thumb screws tightening on to metal washers. It is important that the rubber should be thick enough to prevent the top plate touching the main block, and the holes in the top plate should be cut generously, otherwise the deformation of the plastic which occurs in the incubator makes removal of the top plate very difficult.

After use, the Perspex block, without the rubber washer, and the top plate are separately sterilized by immersing in 1·0% hydrochloric acid. After an hour or so they are washed in running water, the cups being thoroughly cleaned by directing a jet of water into each. After rinsing in distilled water the plates are dried in the incubator.

The rubber washer is now fitted on to the tray, and this and the top plate are separately irradiated with ultra-violet light for a sufficient time to sterilize them.



Text-fig. 2. Scale drawing of plastic tray. Side view.

The tray is stored in a sterile tin, in which it remains until, in a particular experiment, all the cups have been appropriately filled and it is time to fit the top plate. For this purpose the tin is provided with a loosely fitting hinged lid so that during the filling of the cups the lid need only be lifted about 60°, thereby preventing dust particles falling on the plate. The top plate is stored in another sterile tin with a well-fitting lid. Occasionally the plates will require polishing with Perspex polish nos. 1 and 2, after which they are washed with soap and water and rinsed. If mould-infected, they may be disinfected in 10% HCl.

(2) *Rocking machine* (Pl. 12)

A rocking machine of the tilting platform type was used. Although there is no reason to suppose that the exact degree of agitation is important, provided it be sufficient and not too violent, the type used was powered by an electric gramophone motor fitted with a 2·2 gear reduction. This provided 45 tilts a minute and the angle swept was 40°. With this type of rocking machine there is a distinct jerk when the platform changes direction and this facilitates mixing of the contents of

the cups. One of the sides of the platform was made removable and fitted with thumb-screws so that the Perspex tray could be tightly wedged in position.

(3) *Solutions*

(i) *Glucosol* (Parker, 1938):

NaCl	8·0 g.
KCl	0·2 g.
$CaCl_2$	0·2 g.
$MgCl_2.6H_2O$	0·1 g.
$NaH_2PO_4.H_2O$	0·05 g.
Glucose	1·0 g.
Glass-distilled water	1000 ml.

Autoclaved at 15 lb. for 20 min. and stored at +4° C.

(ii) *Modified glucosol*:

NaCl	8·0 g.
$CaCl_2$	0·2 g.
$MgCl_2.6H_2O$	0·5 g.
Glucose	1·0 g.
Glass-distilled water	1000 ml.

Autoclaved at 15 lb. for 20 min. and stored at +4° C.

(iii) *Phosphate buffer* (pH 6·8):

(*a*) Na_2HPO_4 9·473 g.
Sterile glass-distilled water 1000 ml.

(*b*) KH_2PO_4 9·078 g.
Sterile glass-distilled water 1000 ml.

These two solutions are made up separately, then equal parts are mixed and distributed into sterile bottles, which are steamed for 20 min. on three successive days. Stored at +4° C.

(iv) Aqueous penicillin solution containing 10,000 units/ml.

(v) Aqueous streptomycin solution containing 100,000 μg./ml.

(vi) Egg white. Fresh egg white is collected with sterile precautions and centrifuged at 20,000 *g*. for 15 min. The clear supernatant is removed and stored at +4° C. It will keep for months.

(4) *Influenza virus strains*

A strain, PR 8.

A′ strain, Barratt (BAR).

B strain, Lee.

These viruses were stored, sealed in glass ampoules, at −70° C., as infected allantoic fluids mixed with an equal volume of broth.

From time to time the identity of the strains was confirmed by a specific complement fixation test.

(5) *Sera*

Normal and immune ferret sera were stored, sealed in glass ampoules, at −70° C.

DETAILED DESCRIPTION OF THE TECHNIQUE FOR THE TITRATION OF INFLUENZA VIRUS

50 ml. of modified glucosol are mixed with an equal volume of the phosphate buffer (pH 6·8), and to the mixture is added sufficient of the aqueous penicillin to give 10 units/ml., and sufficient of the aqueous streptomycin to give 40 μg./ml.

This solution is now distributed in 1·0 ml. amounts to the 100 cups on the plastic tray, the two bottom cups being left empty.

Fertile hen's eggs containing embryos 9–16 days old (but all of the same age for a particular experiment) are opened and the embryos tipped out and discarded. That part of the chorio-allantoic membrane adhering to the shell is now removed and washed in two changes of glucosol (unmodified). The membranes are then cut up with scissors into pieces about 12×12 mm., the cutting being most convenient if the membranes are floating in a Petri dish of glucosol. Several membranes are cut up in this way, the pieces being pooled in another dish of glucosol until 100 have been accumulated. This is the only laborious part of the technique. With fine sterile forceps one piece of membrane is dropped into each cup.

An ampoule of infected allantoic fluid containing influenza virus is thawed, and 0·1 ml. added to 10 ml. of physiological saline containing 1% egg white. From this initial 10^{-2} dilution, serial tenfold dilutions are made by transferring 1·0 ml. to 9·0 ml. of the same diluent, a fresh pipette being used at each stage.

In a typical titration illustrated in Table 1, the BAR strain was diluted in this way. With a dropping pipette calibrated to deliver 20 mm.^{3} drops, two drops of the 10^{-7} dilution were added to cups *6 to *10 in row 2, two drops of the 10^{-6} dilution to cups *1 to *5 in row 2, two drops of the 10^{-5} dilutions to cups *6 to *10 in row 1 and two drops of the 10^{-4} dilution to cups *1 to *5 in row 1. In an exactly similar way replicates of the titration were made in rows 3, 4; 5, 6; 7, 8; and 9, 10; the same set of virus dilutions being used for all the replicates. The sterile top plate is now removed from its tin, placed in position and screwed down. The whole plastic tray is placed on the rocking machine in an incubator maintained at 36° C., and rocked for about 60 hr. At the end of this time the tray is removed and the top plate unscrewed. With a pair of fine forceps the pieces of membrane are removed from the cups, each piece, as it is removed, being plunged into a beaker of boiling saline, the hot forceps being then cooled by dipping into cold saline. To each cup is added a drop of 4% washed chicken erythrocytes, the drop being 40 mm.^{3} from a calibrated dropping pipette. To ensure that the erythrocytes are neither auto-agglutinable nor inagglutinable a positive and a negative control are included in the cups below the tenth row.

The erythrocytes are mixed with the fluid in the cups by rapid to and fro movement of the tray on the bench. After about 1 hr. the result is read by observing the characteristic pattern of the red cells. In the majority of cups the result will be clearly either positive or negative. Occasionally the agglutination is only partial, in which case it is recorded as positive.

The result of one such titration is shown in Table 1. By combining the results

of the replicates the ID 50 (the dose at which exactly half of a large number of replicates would be infected) has been estimated by the probit method (Finney, 1947), and expressed as the volume in mm.^{3} of the seed virus.

Critique of the technique

(i) *Suspending fluid*

Tyrode was found to be unsatisfactory, since it did not, even with added CO_2 in the immediate gas phase, possess sufficient buffering power. Consistent multiplication of virus was obtained by omitting the bicarbonate and diluting

Table 1. *Tissue suspension titration of influenza A′ (BAR) virus*

(i) *Plan*

	*1 *2 *3 *4 *5	*6 *7 *8 *9 *10
1	10^{-4}†	10^{-5}
2	10^{-6}	10^{-7}
3	10^{-4}	10^{-5}
4	10^{-6}	10^{-7}
5	10^{-4}	10^{-5}
6	10^{-6}	10^{-7}
7	10^{-4}	10^{-5}
8	10^{-6}	10^{-7}
9	10^{-4}	10^{-5}
10	10^{-6}	10^{-7}

(ii) *Result*

	*1	*2	*3	*4	*5	*6	*7	*8	*9	*10
1	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	−	−	−	−	−
3	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	−	+	+	−	−
5	+	+	+	+	+	+	+	+	+	+
6	+	+	+	−	+	−	−	+	−	−
7	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	−	−	−	−	−
9	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	−	+	−	−	+

(iii) *Computation*

Initial virus dilution	Inoculum (mm.^{3} of seed $\times 10^{-6}$)	Result +	Result −
10^{-4}	4000	25	0
10^{-5}	400	25	0
10^{-6}	40	24	1
10^{-7}	4	5	20

ID 50 = $8{\cdot}24 \times 10^{-6}$ mm.^{3}
See Table 6 (iii).

† Initial virus dilutions.

glucosol salt solution with an equal volume of isotonic (M/15) phosphate buffer. A pH of 6·8 was chosen because at this level the phosphate mixture has its maximum buffering capacity, and small variations around this point had no effect on virus multiplication. No multiplication of virus occurred at pH 6 or 8. The phosphate buffer could not be replaced by a veronal buffer.

The KCl could be omitted from the glucosol because there were ample potassium ions in the buffer. The magnesium ion was proved to be essential, and rather more consistent virus multiplication was obtained when the $MgCl_2.6H_2O$ salt was increased five times. The calcium ion was essential but could not be increased

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because addition of more $CaCl_2$ caused a precipitate to develop. The glucose in the amount used was essential, but the medium was not improved by increasing its concentration.

Although virus multiplication has been used as the guide in designing the suspending fluid it is probable that the main effect of modification of the saline medium is on the cells of the chorio-allantoic membrane, rendering them more or less able to support virus multiplication.

It was considered desirable to keep the suspending fluid as simple as possible so as to avoid the presence of virus inhibitors. Although it is impossible to say that the suspending fluid adopted is the best that can be devised, it would seem to approximate to the optimum for this particular virus cycle; for the substitution of a complex medium like chick amniotic fluid, which is a complete medium for the actual culture of chick cells (Grossfeld, 1949), gave effectively the same virus ID 50 as is regularly obtained using the buffered salt solution.

Virus dilutions were made in 1·0% egg-white saline, so that the final concentration of egg white in the suspending fluid in the cups was 0·04%. Under the conditions of the test, this amount of egg white did not materially affect the sensitivity of the subsequent haemagglutination test. Egg white, even in a concentration of 1·0%, does not improve the suspending fluid. Broth and mammalian sera were avoided as virus diluents because, even in small amounts, they inhibited virus multiplication.

The presence of some antibiotic is essential, for bacterial infection quickly destroys the membrane. Ten units of penicillin per ml. are sufficient, but as an added safeguard 40 μg./ml. of streptomycin are included, as influenza virus multiplication is not adversely affected thereby. Occasionally after incubation a cup on the plastic tray will be found to be supporting a mould. No special precautions have been taken to avoid this mishap because it occurs so infrequently, and when it has occurred in cups in which virus growth was expected there seemed to have been no interference with virus multiplication.

(ii) *Tissue cells*

Chick embryos 9–16 days old are suitable; chorio-allantoic membranes derived from the youngest embryos behave in the same way as membranes from the oldest.

Before 9 days the allantoic membranes are too small to be economically employed. After 16 days the suspending fluid which has contained the pieces of infected membrane becomes inconveniently mucoid. In most of the tests chorio-allantoic membranes from 14-day embryos have been preferred.

Usually only that portion of the chorio-allantoic membrane is used which adheres to the shell when the embryo is tipped out of the egg through a rent in the membrane underlying the air sac. That part of the chorio-allantoic membrane immediately around the attachment to the albumin sac is cut off and discarded, since it looks oedematous and obviously different in appearance from the rest of the membrane. Washing the membrane in glucosol does not harm it.

As an average, twenty-five pieces of membrane are obtained from each egg,

though the actual number will, of course, depend on the size of the membrane, the range being 15–45 pieces.

Each piece is about 12×12 mm., but as they are estimated by eye they will vary considerably. However, the size has been chosen to allow a wide margin of safety. Four times as much tissue shows little, if any, improvement; one-eighth of the amount does reduce the sensitivity (Table 2). But these differences in size are considerably greater than will occur among the standard pieces.

Although for this technique it is essential that the membrane shall be in one piece, it is interesting to observe that mincing the membrane with scissors

Table 2. *Effect of the size of the piece of chorio-allantoic membrane on the sensitivity of the tissue suspension for virus titration*

Inoculum (mm.³ of seed (BAR) $\times 10^{-6}$)	Positive cups (out of five)			
	Standard pieces area approx. 150 mm.²	$\times 4$ Standard area	$\times \frac{1}{8}$ Standard area	Standard pieces, minced
4000	5	5	5	5
40	3	4	0	0
4	0	1	0	0
0·4	0	0	0	0

Table 3. *Variability of individual chorio-allantoic membranes*

Membrane	Inoculum (mm.³ of seed $\times 10^{-6}$ (BAR))			
	133	44	15	5
1	5†	5	1	0
2	5	3	4	1
3	5	4	0	0
4	5	3	3	1
5	5	5	3	0

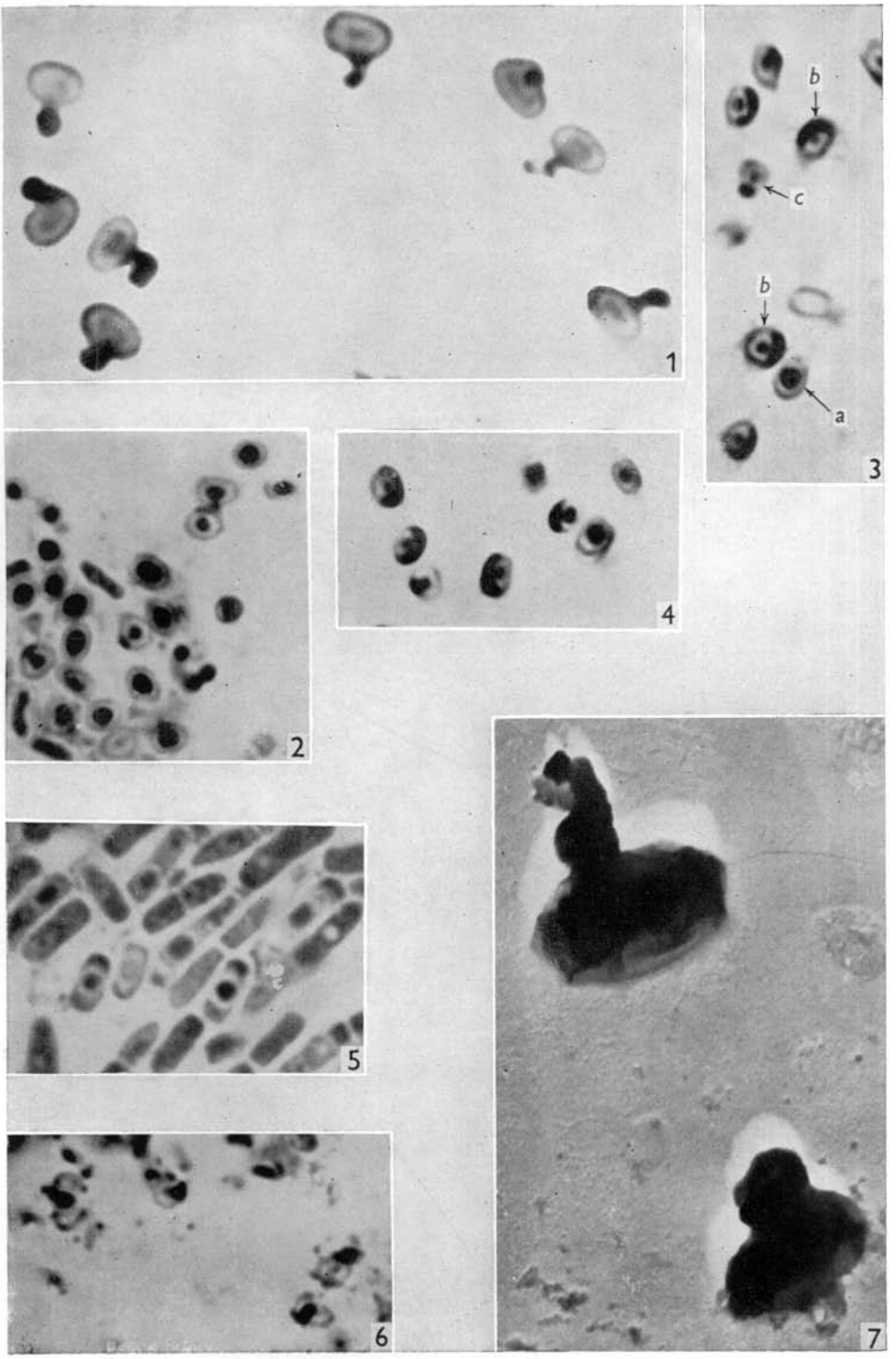
† Positive cups (out of five).

Assuming a common slope of 1·27, the slopes of the individual probit lines differ significantly:

$$\chi^2 = 9{\cdot}86, \text{ for 4 d.f., } 0{\cdot}02 < P < 0{\cdot}05.$$

reduces the sensitivity of the system for virus titration (Table 2). If the membranes are ground in a mortar or fragmented in a Waring Blendor, no virus multiplication can be detected.

It was convenient to pool the pieces from a number of chorio-allantoic membranes so that they were distributed at random over the tray. If the individual membranes had varied very much in their ability to support virus multiplication, this random distribution would have contributed unpredictably to the slope of the regression line used in calculating the ID 50, and it would have been better to have located the membranes on the tray so that their variability could have been allowed for in the final calculation. However, the individual chorio-allantoic membranes do not differ widely in sensitivity. Table 3 illustrates a titration carried out five times on pieces from five chorio-allantoic membranes.



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Assuming a common slope of 1·27, the individual ID 50's do not differ significantly. The slopes of the individual probit lines, however, do differ significantly: $\chi^2 = 9{\cdot}86$, for 4 degrees of freedom; $0{\cdot}02 < P < 0{\cdot}05$. However, in the design of the technique it was decided to ignore this slight variability in individual chorio-allantoic membranes.

The suspension and incubation of the pieces of membrane in the buffered salt solution will inevitably lead to the progressive deterioration and finally to the death of the cells composing the membrane. Fortunately, the rate at which this occurs is slower than the rate of influenza virus multiplication, so that several growth cycles can take place before the cells become useless.

Thus it was found that after 24 hr. rocking in the cups before the addition of virus dilutions, pieces of a 14-day membrane were apparently as well able to support virus multiplication as when freshly harvested (Table 4).

Membranes kept in the buffered salt solution for 4 days at 36° C. (not rocked) were not able to support virus multiplication; membranes stored in the same way

Table 4. *Deterioration of the chorio-allantoic membrane in the buffered salt solution at* 36° C.

Inoculum (mm.³ of seed (BAR) × 10^{-6})	Positive cups (out of five) Membrane infected		
	Immediately	After 24 hr. at 36° C.	After 96 hr. at 36° C.
4000	5	5	0
400	5	5	0
40	4	5	0
4	2	1	0

but at +4° C. allowed virus multiplication in two of five cases. After storage in the buffered salt solution for 14 days at +4° C. they were no longer capable of supporting virus multiplication.

The deterioration of the membrane during the experimental period is seen very clearly histologically.

6 μ sections of pieces of membrane were prepared by a new and very rapid technique which will be fully described elsewhere (Fulton, 1951).

The sections were stained with Lillie's buffered azure-eosin stain (1948) or with Heidenhain's iron haematoxylin. After 24 hr. in the cups the membranes showed little deterioration, but at the end of the 60 hr. period the membrane, even when not infected with virus, was beginning to break down and to lose its epithelium on both sides (Pl. 13). This epithelial loss is usually more marked when the membrane has been infected with virus, but the difference is not sufficiently striking for it to be used as a way of detecting the end-point of a virus titration.

Although the chorio-allantoic membrane is the most convenient source of tissue from the developing egg, it is possible to use amniotic membrane, though it does not give better results. Very low haemagglutinin titres were obtained when pieces of yolk sac were used as a source of cells.

(iii) *Temperature*

No significant difference was observed over the range 35–38° C.; 36° C. was adopted as standard. No virus multiplication was obtained at 41° C. and very little at 30° C.

(iv) *Time*

In the initial experiments the suspending fluid was titrated for haemagglutinin at the end of 48 hr. Although this is probably a just sufficient period, a small increase in titre was often observed if the experiment was continued another day. In consequence about 60 hr. was chosen as a convenient time allowing a fair margin of safety. With small inocula no haemagglutinins are demonstrable during the first 24 hr.

In the preliminary work when whole membranes floating in 20 ml. of fluid in a 250 ml. flask were used, it was found to be essential to rock the flasks during incubation. On the plastic tray the relative volumes are quite different, and some virus multiplication does occur if the plate is incubated but not rocked. However, despite careful initial mixing, the sensitivity of the system for virus titration is reduced about a hundredfold.

A sample of infected allantoic fluid containing BAR virus was found by the standard technique to have an ID 50 of $3{\cdot}41 \times 10^{-6}$ mm.^{3} with 95 % fiducial limits at $1{\cdot}84 \times 10^{-6}$ and $5{\cdot}63 \times 10^{-6}$ (cf. Table 6 (*b*) (v)). By the same technique, except that the tray was not rocked, the ID 50 was 1055×10^{-6} mm.^{3} (474×10^{-6} and 2353×10^{-6}). The ratio of the two ID 50's is 309·1 (122·3 – 781·3).

It was later found not to be strictly necessary to rock the plastic tray during the whole of the incubation period, for rocking could be discontinued after the first 24 hr. without affecting the titre. With this modified technique, the same virus suspension had an ID 50 of $3{\cdot}26 \times 10^{-6}$ mm.^{3} ($1{\cdot}25 \times 10^{-6}$ and $6{\cdot}66 \times 10^{-6}$). Compared with the standard technique, the ratio of the ID 50's is 1·05 (0·41 – 2·70).

Methods of detecting the end-point of the virus titration

(i) The titration method described is based on the development of virus haemagglutinin. Even with the largest virus inocula used, the haemagglutinin measured at the end of the incubation period cannot be the actual virus particles added as seed. For the final dilution of the seed virus would be $10^{\overline{4}\cdot 6}$ (equivalent to inoculum of 400×10^{-3} mm.^{3} of seed), and the haemagglutinin titre of the seed was only $10^{\overline{3}\cdot 2}$. In the majority of the experiments the lowest dilution of the seed virus at the start of the incubation period was $10^{\overline{6}\cdot 6}$ (equivalent to inoculum of 4000×10^{-6} mm.^{3} of seed). It is easy to show that the rise of haemagglutinin is associated with a rise of infective virus, so that it is a valid inference in this case to suppose increase in haemagglutinin to indicate virus multiplication. In a particular experiment using an inoculum of 4000×10^{-6} mm.^{3} of seed virus, even supposing that all the virus added remained viable during the course of the experiment, there was a 10,000-fold increase in infective virus. The haemagglutinins which develop usually have a titre after 60 hr. of about 1/100 measured with chick erythrocytes in a final concentration of 0·25 %. The highest titre

recorded has been 1/512, and with small inocula close to the ID 50 the titres are often lower. A typical distribution is illustrated in Table 5.

With small inocula close to the ID 50 those cups which are negative by haemagglutination do not contain infective virus detectable by inoculation into eggs. Thus the substitution of an infectivity titration for the haemagglutination titration would not materially increase the sensitivity of the test.

If virus is added to cups containing suspending fluid but no tissue, there is a rapid fall in its infectivity, 90% of the seed virus becoming non-infective in 24 hr. under the experimental conditions. This fall in infective virus in the suspending fluid is not due to the adsorption of virus on to the Perspex.

(ii) It is possible to measure the end-point of the virus titration by detecting the presence or absence of specific complement-fixing antigen in the suspending fluid after the period of incubation, the seed virus being far too dilute to be measurable. The technique will not, however, be discussed here in detail since it is not of immediate importance in this particular instance, the influenza virus being so readily titrated by haemagglutination. Briefly, use is made of the apparatus for complement-fixation already described by Fulton & Dumbell (1949).

Table 5. *Distribution of haemagglutinin titres in relation to virus inoculum*

Inoculum (mm.^{3} of seed (BAR) $\times 10^{-6}$)	No. of replicates with haemagglutinin titre of			
	<1	1–20	40	$\geqslant 80$
4000	0	1	0	4
400	0	2	1	2
40	1	4	0	0
4	5	0	0	0

For the titration of a complete tissue suspension tray only three sheets of the complement fixation apparatus are required.

On sheet 1 a drop of inactivated immune serum is added to each square, the serum being diluted to contain 5 units of antibody.

On sheet 2 a drop of inactivated normal serum of the same species and at the same dilution is similarly added to each square.

The squares on both sheets now receive a drop of guinea-pig serum diluted to contain 2 units of complement, and finally a drop of the suspending fluid from each cup is added to an appropriate square on both plates. The third sheet is used for complement and serum controls, but there is no need to include antigen controls, since any anti-complementary effect will be detected on the sheet containing the normal serum. The rest of the test is carried out in the way which has already been described in detail in the paper referred to above.

Although extensive comparisons have not yet been made, an excellent correlation has been observed between the estimate of virus multiplication by haemagglutination and by complement-fixation.

In general, a slightly lower apparent virus titre may be expected, since the complement-fixation test with the 600 S antigen is at least ten times less sensitive than the haemagglutination test as a measure of the concentration of elementary bodies.

Comparison of in ovo *and tissue suspension titration of influenza virus*

A stored pool of allantoic fluids infected with BAR virus was titrated in batches of 10-day embryos, first in tenfold dilution steps and then, over the critical range, in fivefold dilution steps. The virus dilutions were made in 1·0% egg-white saline, and 40 mm.^{3} of the appropriate dilutions were inoculated into the allantoic cavity

Table 6. *Comparison of* in ovo *and tissue suspension titration of influenza A′ (BAR) virus*

(*a*) *In ovo* titration

	Inoculum (mm.^{3} of seed (BAR) $\times 10^{-6}$)	Result +	Result −	ID 50 with 95% fiducial limits
(i)	40	9	1	$0{\cdot}48 \times 10^{-6}$
	4	10	0	$(0{\cdot}19 \times 10^{-6}$–$1{\cdot}24 \times 10^{-6})$
	0·4	6	4	
	0·04	0	10	
(ii)	4	16	1	$0{\cdot}20 \times 10^{-6}$
	0·8	16	1	$(0{\cdot}10 \times 10^{-6}$–$0{\cdot}36 \times 10^{-6})$
	0·16	7	10	
	0·032	1	15	

Common slope of probit lines ± standard error = 1·423 ± 0·232.
Weighted mean ID 50 for (i), (ii) combined = $0{\cdot}26 \times 10^{-6}$ $(0{\cdot}16 \times 10^{-6}$–$0{\cdot}43 \times 10^{-6})$.

(*b*) Tissue suspension titration

(iii)	4000	25	0	$8{\cdot}24 \times 10^{-6}$
	400	25	0	$(4{\cdot}63 \times 10^{-6}$–$14{\cdot}06 \times 10^{-6})$
	40	24	1	
	4	5	20	
(iv)	400	5	0	$17{\cdot}36 \times 10^{-6}$
	200	4	1	$(8{\cdot}62 \times 10^{-6}$–$33{\cdot}76 \times 10^{-6})$
	100	5	0	
	50	4	1	
	25	3	2	
	12·5	1	4	
	6·25	2	3	
	3·125	1	4	
(v)	400	25	0	$3{\cdot}41 \times 10^{-6}$
	80	24	1	$(1{\cdot}84 \times 10^{-6}$–$5{\cdot}63 \times 10^{-6})$
	16	23	2	
	3·2	12	13	

Common slope of probit lines ± standard error = 1·742 ± 0·249.
Weighted mean ID 50 for (iii), (iv) combined = $11{\cdot}09 \times 10^{-6}$ $(7{\cdot}34 \times 10^{-6}$–$16{\cdot}77 \times 10^{-6})$.
Ratio of combined ID 50's = 42·71 (22·33 to 81·70).

in the usual way. After incubation at 36° C. for 2 days the eggs were chilled and the allantoic fluids separately tested for the presence or absence of haemagglutinin. The results are shown in Table 6 (*a*). The probit lines fitted to the two series separately did not differ significantly either in position or slope. The ID 50 was estimated separately for each series using a common slope of 1·423; and, finally, a combined value was obtained by weighting each of the two estimates of the log ID 50 in inverse proportion to its estimated variance. This final estimate of the

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ID 50 is $0{\cdot}26 \times 10^{-6}$ mm.^{3} of seed virus, with 95 % fiducial limits at $0{\cdot}16 \times 10^{-6}$ and $0{\cdot}43 \times 10^{-6}$.

The same pool of allantoic fluids infected with BAR virus was titrated twice by the standard tissue suspension technique, first in tenfold dilution steps, and then, over the critical range, in twofold dilution steps. These titrations were carried out at the same time as the *in ovo* titrations. Later the tissue suspension titration was made again, in fivefold dilution steps. The results are shown in Table 6 (*b*). The probit lines fitted to the three series did not differ significantly in slope. Using a common slope of 1·742 the ID 50 was estimated separately for each series. Since the titration in fivefold dilution steps was done at a later date and differed significantly from the other two in position, for the comparison with the *in ovo* titration a combined value for the ID 50 was obtained from the first two estimates. This final estimate of the ID 50 is $11{\cdot}09 \times 10^{-6}$ mm.^{3}, with 95 % fiducial limits at $7{\cdot}34 \times 10^{-6}$ and $16{\cdot}77 \times 10^{-6}$.

From the comparisons the *in ovo* method is estimated to be 42·71 times more sensitive as a method of detecting egg-adapted influenza virus than the tissue suspension method, the 95 % fiducial limits for this ratio being 22·33 and 81·70.

However, as a means of estimating the titre of a virus suspension, the accuracy of the estimate will depend on the number of eggs or the number of cups used at each dilution level. From a consideration of the slope of the regression line in the combined *in ovo* titrations, and the slope of the regression line in the combined tissue suspension titrations, it is estimated that K pieces of membrane are equivalent to one egg, where K is estimated at 0·67.

This factor is, in fact, the ratio of the squares of the common slopes of the two sets of probit lines, 1·423 and 1·742; in consequence, it is subject to sampling error. The 95 % fiducial limits are approximately 0·27 and 1·57.

The technique of a serum neutralization test

If unheated mammalian sera, even in high dilution, are added to the tissue suspensions, no virus multiplication can be detected. Sera which have been inactivated by heating at 56° C. for 30 min. are less inhibitory, but even so a final concentration of serum of 10^{-2} is always, and a final concentration of 10^{-3} is sometimes, inhibitory.

It is therefore impracticable to titrate neutralizing antibodies by adding serum directly to the cups; even if the sera had not been so inhibitory to virus multiplication, their presence in the cups would mask the subsequent haemagglutination test for virus.

It was observed that when pieces of membrane were suspended in fluid containing a high concentration of virus, the cells quickly became infected, so that if the membrane was removed after a short exposure, washed and placed in fresh suspending fluid not containing virus, the virus in the membrane would multiply and build up a sufficient concentration of virus in the suspending fluid to be detected by haemagglutination.

A particular example of this transfer technique is illustrated in Table 7. To each of three screw-capped bottles of 20 ml. capacity was added 5 ml. of suspending

fluid. Another set of three similar bottles was prepared each containing 5 ml. of suspending fluid with inactivated guinea-pig serum at a final concentration of 10^{-1}.

To the first bottle of each set 2000×10^{-3} mm.^{3} of PR 8 virus suspension were added ($= 400 \times 10^{-3}$ mm.^{3}/ml.).

To the second bottle of each set 200×10^{-3} of the seed virus, and to the third bottle of each set 20×10^{-3} of the seed virus were added. The ID 50 of the seed virus suspension was approximately $0{\cdot}4 \times 10^{-3}$ mm.^{3}.

The suspensions were left to stand at room temperature for half an hour, and five pieces of membrane were then added to each bottle. The six bottles were transferred to the incubator at 36° C. and rocked for 1 hr. At the end of this time

Table 7. *A serum neutralization test*

(*a*) Infection of chorio-allantoic membranes by exposure for 1 hr. to influenza virus in the presence of and in the absence of inactivated normal guinea-pig serum.

Inoculum (mm.^{3} of seed $\times 10^{-3}$ per ml.) PR 8 virus	Positive cups (out of five): Virus without serum	Positive cups (out of five): Virus with 10^{-1} normal guinea-pig serum
400	5	5
40	5	5
4	4	2

(*b*) Specific serum neutralization test with an inoculum of 400×10^{-3} mm.^{3} of seed virus per ml. Exposure of membranes to serum/virus mixture for 1 hr.

Inactivated ferret serum 10^{-2}	Positive cups (out of five): PR 8 virus	Positive cups (out of five): Lee virus
Normal	5	5
Immune PR 8	0	5
Immune Lee	5	0

the suspending fluid in the bottles was removed with a pipette and an equal volume of glucosol substituted. The plastic tray had already been prepared so that the cups contained 1 ml. of fresh suspending fluid. With fine forceps the pieces of membrane were then transferred separately to appropriate cups.

The tray was rocked for 60 hr. in the incubator, and the presence or absence of haemagglutinin estimated in the usual way. The result is shown in Table 7 (*a*).

An inoculum of 4×10^{-3} mm.^{3} of seed virus/ml. is clearly insufficient to ensure infection of the membranes in the hour allowed. For the main test an inoculum of 400×10^{-3} mm.^{3}/ml. was chosen.

The result of one titration of neutralizing antibodies, using this transfer technique, is shown in Table 7 (*b*). A PR 8 and a Lee ferret antiserum were tested at a dilution of 10^{-2} with PR 8 virus and a pool of Lee virus of comparable titre. A clear-cut and specific virus neutralization was obtained.

DISCUSSION

Once the apparatus has been assembled, the tissue suspension technique for influenza virus titration is probably simpler than *in ovo* titration, and the accuracy of the estimate of an ID 50 can be increased to any reasonable extent by multiplying the number of replicates.

Serum neutralizing antibodies can be titrated, and the technique may be useful in the study of other virus inhibitors and for the analysis of virus growth cycles, though the constants of the cycle will probably be different from those disclosed in living chick embryos in the beautiful experiments of the Henles (1949).

Theoretically, the titration method proposed seems capable of considerable improvement if the membranes could be preserved in a healthy state for a longer time, and if the infectivity of the seed virus did not fall so rapidly. These factors are, however, perhaps of little importance in the present application, owing to the very rapid growth cycle of the influenza virus.

The technique should be adaptable, without much modification, to the titration of egg-adapted strains of Newcastle disease virus and of Fowl Plague virus. Mumps virus, on the other hand, has a rather slow growth cycle, so that it may be necessary in this case to buttress the health of the membranes.

If complement-fixation is used for detecting the titration end-point, many other viruses come within the range of the method, modified to suit special conditions. Nor is it necessary to confine attention to tissues from chick embryos, the only requirement being that the susceptible cells shall be arranged as a flat membrane. It is possible that mammalian amnia or embryo intestine will be suitable.

In all these examples, however, the essential point is that virus multiplication shall be considerable and that the virus particles or some soluble antigen shall be dispersed in high titre in the suspending fluid. In cases when this does not occur it may still be possible to detect virus multiplication by the antigenic analysis of the pieces of membrane.

SUMMARY

A new method of titrating the infectivity of egg-adapted influenza virus suspensions is described.

For this purpose a plastic tray has been made, drilled with one hundred conical cups. In each cup a small piece of chorio-allantoic membrane from a chick embryo is suspended in a buffered salt solution. To these surviving tissue suspensions virus dilutions are added, and the tray is put in an incubator and rocked mechanically for 60 hr.

In those cups in which the inoculum has been sufficiently large, the membrane becomes infected by the virus which multiplies within the cells and, bursting out, builds up by infection of other cells a high concentration of virus in the suspending fluid. The virus multiplication is detected by removing the pieces of tissue and adding to the cups a drop of washed chicken erythrocytes. The presence of virus in the cup is shown by the pattern of the sedimented erythrocytes, and virus multiplication is inferred because the original seed virus is too dilute to be detectable by haemagglutination.

The method has been compared with *in ovo* titration. As a means of detecting egg-adapted infective virus, the tissue suspension is between 20 and 80 times less sensitive; as a titration system, however, approximately one cup is equivalent to one egg, so that by using sufficient replicates accurate assays of infective virus can be made.

The technique is readily adaptable to the titration of serum-neutralizing antibodies.

In addition to haemagglutination, the infectivity end-point of the titration can also be estimated by a specific complement fixation test, and it is suggested that, with modifications, the new technique may be applicable to the titration of a large number of other viruses.

Miss Irene Allen helped in the computation of the probit lines. The rocking machine was designed for us some years ago for another purpose by Dr E. J. H. Schuster. The model used in this work was constructed by Mr H. D. Dawson, who also drilled the Perspex trays. Mr W. T. Bush photographed the apparatus and also prepared the photomicrographs.

REFERENCES

BERNKOPF, H. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 680.
FINNEY, D. J. (1947). *Probit Analysis.* Cambridge University Press.
FULTON, F. (1951). *J. Path. Bact.* (in the Press).
FULTON, F. & DUMBELL, K. R. (1949). *J. gen. Microbiol.* **3**, 97.
GROSSFELD, H. (1949). *Proc. Soc. exp. Biol., N.Y.*, **71**, 475.
HENLE, W. & HENLE, G. (1949). *J. exp. Med.* **90**, 1.
LAHELLE, O. & HORSFALL, F. L. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 547.
LILLIE, R. D. (1948). *Histopathologic Technic.* Philadelphia: The Blakiston Company.
PARKER, R. C. (1938). *Methods of Tissue Culture.* New York: Hoeber.
SALK, J. E. (1948). *Science*, **108**, 749.
WELLER, T. H. & ENDERS, J. F. (1948). *Proc. Soc. exp. Biol., N.Y.*, **69**, 124.

EXPLANATION OF PLATES 12 AND 13

PLATE 12

Rocking machine with plastic tray on platform (one side of the platform removed).

PLATE 13

6μ sections of uninfected chorio-allantoic membranes stained with azure-eosin. Magnification: $\times 150$.

Fig. 1. Immediately after removal from egg.

Fig. 2. After 24 hr. in the buffered salt solution.

Fig. 3. After 60 hr. in the buffered salt solution. The membrane appears to be oedematous, and has already begun to break down.

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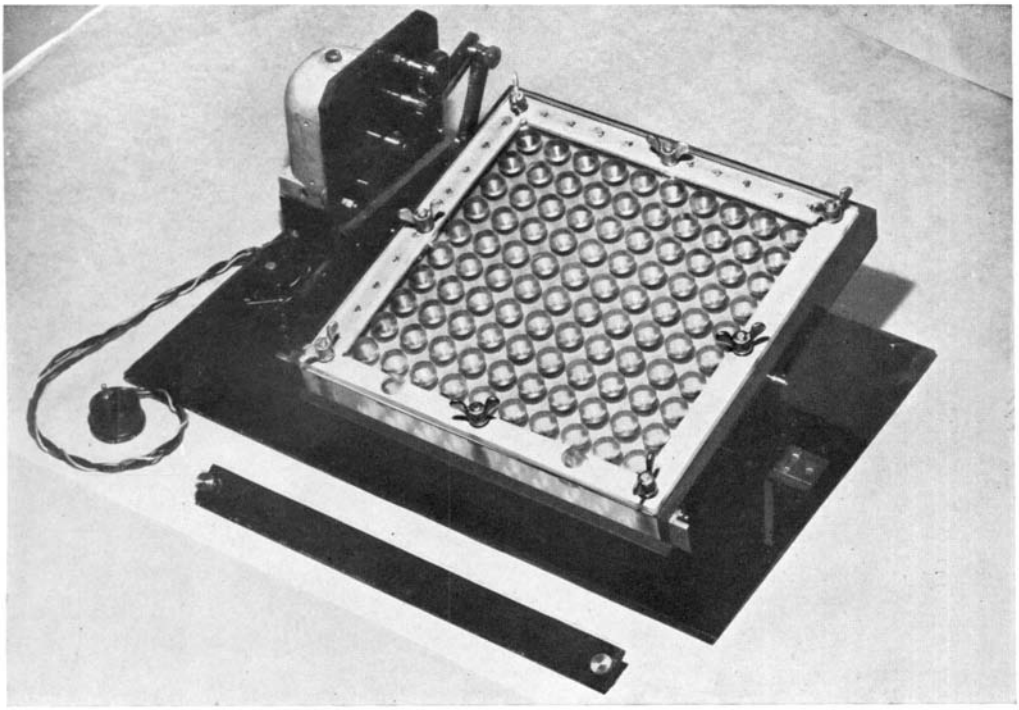

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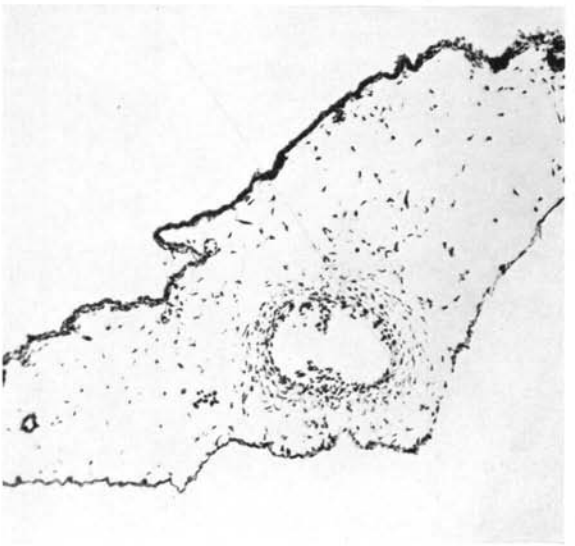


Fig. 1.

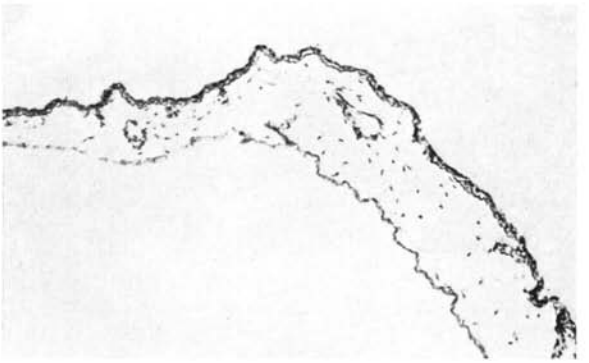
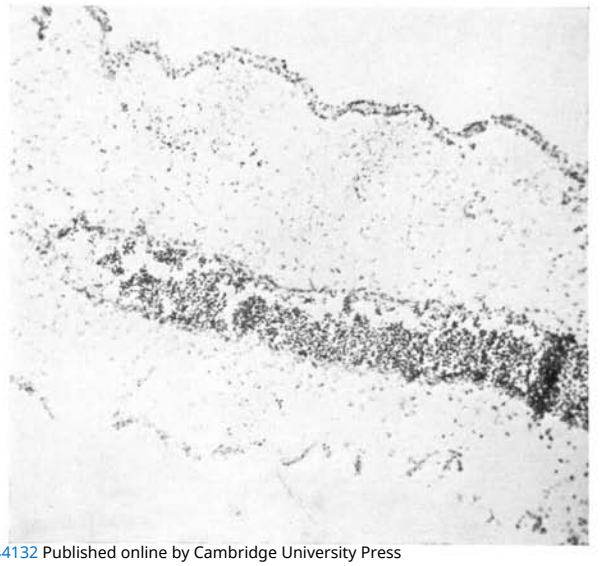


Fig. 2.



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