THE TECHNIQUE OF QUANTITATIVE CHORIOALLANTOIC VIRUS TITRATION

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Received for publication, November 10, 1941

Although the quantitative "pock counting" method of titrating a wide variety of viruses on the chorioallantois has been used in this laboratory for the last six years, there has been very little application of the method elsewhere. Schaffer and Enders (1939) studied the inactivation of herpes virus by immune serum along these lines and found the method satisfactory. Nelson (1938) has used the technique in roughly quantitative form for work with vaccinia and variola viruses. Haagen (1940) on the other hand considers that the method is quite unsuited for use as a general method for vaccinia virus titration.

In the course of work on the photodynamic inactivation of herpes and vaccinia viruses with methylene blue we have attempted to improve several aspects of the technique. In essentials our present methods are the same as those described by Burnet in 1936 but attention to details has resulted in a considerable improvement in the membranes obtained.

In our experience there is only one important cause for unsatisfactory results; this is haemorrhage on the inoculated surface of the chorioallantois. A large haemorrhage will cause death of the embryo within 24 hours while smaller ones are almost invariably associated with nonspecific lesions of the ulcer type as described in the 1936 monograph. There seems to be an irregular seasonal variation in the readiness with which haemorrhages occur, the late summer being generally the most unsatisfactory period. There are also small-period fluctuations, some weeks' supplies of eggs giving noticeably more satisfactory results than others. We have not been able to control the occurrence of these variations and can only suggest tentatively that they may be related to the nutrition of the laying hens; the possibility that the level of vitamin K may be responsible for the variation might be worth studying.

Irrespective of the intrinsic character of the eggs supplied, the incidence of haemorrhage is largely determined by the care with which the various manipulations incidental to the inoculation are carried out. Haemorrhage may result from the trauma of a drill cut involving the shell membrane, from the turning in of a pointed angle as the triangle of shell is removed or from damage produced when the slit is made in the shell membrane in the first stage of inoculation. The capillaries from which haemorrhage occurs lie very superficially in direct contact with the inner surface of the shell membrane and haemorrhage may also be produced in the process of separating the chorioallantois and shell membrane. The only important modification of technique which we have recently introduced is designed to minimize damage during separation. This is to draw in a drop of sterile saline to act as a fluid wedge and make the falling

away of the chorioallantois under the influence of suction and gravity proceed more easily and smoothly. Direct examination of the chorioallantois in eggs drilled with a large opening for amniotic inoculations shows that with this technique the chorioallantois separates cleanly with no trace of haemorrhage in at least 80 per cent of instances where the technique has been correctly followed and the eggs are in a satisfactory condition. Using the former technique in which the chorioallantois was drawn away from the shell membrane without the use of saline, a certain amount of haemorrhage is almost invariable.

DETAILED DESCRIPTION OF CURRENT TECHNIQUE

Drilling

A "Vulcarbo" disc No. 8 is used with a dental engine and three cuts through the shell are made to form an equilateral triangle 1.0 to 1.2 cm. along each side. Care should be taken to avoid the slightest damage to the shell membrane and our routine is to make the cut deeper at the angles than at the middle of the sides of the triangle. A minor point of convenience is to place the triangle so that with the air space end of the egg to the right, one side of the triangle runs parallel to the long axis of the egg and the two other sides converge toward the observer. This disposition of the triangle facilitates the reflection of the flap of shell membrane during inoculation. The drilling is completed by making a small cut through the compact layer of the shell over the air space. After drilling, the eggs are returned to the incubator. They should be inoculated within two hours of being drilled. Inflammatory changes develop rapidly in the chorioallantois beneath the drill marks and within less than 24 hours the membrane becomes adherent to the shell membrane at these points.

Inoculation

The equipment used is extremely simple, a plasticine stand to support the egg about 3 cm. above bench level, a straight triangular cutting-edge needle mounted in a suitable handle, a medium-sized nail or some similarly pointed object to make the opening into the air sac, capillary pipettes and rubber teats.

An opening is first made into the air sac with the nail point. With the needle each corner of the triangle is gently prised up a little until it is clear that the triangle of shell will lift off easily. If this precaution is not taken it is easy to lift one side of the triangle and force the sharp angle opposite through the shell membrane. In the middle of the area of shell membrane so exposed a drop of sterile bland fluid (normal saline with 0.044 per cent $CaCl_2$ is usually used) is placed. Through this drop a slit is made in the fibres of the shell membrane and enlarged sufficiently to be sure that the saline has come in contact with the upper surface of the chorioallantois. The details of making the slit are as follows: The needle point should be only moderately sharp and should contact the shell membrane at an angle of about 45°. It is pressed slightly downwards until the point engages the fibres of the shell membrane and then raised so as to split the membrane along the direction of its fibres. The edge of

the slit should be raised sufficiently to extend the opening about 3 mm. and to allow a glimpse of the chorioallantois. Haemorrhage should be completely absent. The egg is now put aside for a minute or two while others are similarly dealt with. During this period the saline begins the work of gently separating the chorioallantois from the shell membrane. The next stage is to complete the formation of the artificial air space by suction with a rubber teat over the opening into the natural air sac. This should be done very gently and the suction stopped as soon as the chorioallantois is seen to fall away from the shell membrane. Once started, the weight of the egg contents will complete the process.

The slit is now enlarged with the needle and a flap made by cutting with the edge of the needle along the longitudinal edge of the triangle. If this flap of moist shell membrane is turned forward and pressed on to the shell it will adhere and leave a suitable opening for inoculation. The inoculating pipette is a capillary pipette with rubber teat, the capillary having been previously graduated at 0.05 ml. with a weighed drop of mercury. The pipette is held vertically and the inoculum deposited on the chorioallantois without touching shell or shell membrane.

Sealing

After a trial of transparent adhesive tape for sealing the opening we have reverted to the original method of using a coverslip on a rim of vaseline-paraffin mixture. The only technical point worth noting is to avoid allowing the melted paraffin to encroach on the edge of the opening. If the shell membrane—shell junction is infiltrated with paraffin right around the triangle, the air in the artificial air space is absorbed during the subsequent period of incubation.

Removal and examination of membranes

After the required period of incubation the eggs are opened and the chorioallantoic membranes removed. A six-inch petri dish with a suitably moulded pad of wet cotton wool to support the egg is suitable for the purpose. The coverslip is removed and the shell broken away to the level of the fallen chorioallantois. Any shreds of shell membrane are clipped away so that the edge of the area over which the inoculum was spread can be clearly seen. The chorioallantois is now removed with scissors, the cuts being made several millimetres outside the margin of the inoculated area. The membrane is dropped into a dish of 10 per cent formalin in saline and rinsed of blood clot, etc. Then it is spread out on a sheet of black photographic paper kept moist with formol saline. A bent glass spreader is a useful adjunct to the forceps in spreading the membranes satisfactorily. The membranes are left 5 to 10 minutes before being transferred to half petri dishes for examination. After fixation in the stretched condition they will lie flat in saline and the lesions can be examined and counted over a black background. For detailed examination the most convenient set up we have found is to spread the wet membrane out on a square of glass (lantern slide cover) and examine it with a hand lens against a

black background. We have used a petri dish lid painted black as a suitable support and background for examination. A certain amount of light coming in from the side and below makes it much easier to study the membrane than when it is spread directly on a black surface. The larger bloodvessels will usually provide a suitable frame of reference for counting.

In counting the numbers of specific lesions a certain amount of experience of the virus under study is needed in order to become familiar with the various appearances which the lesions may take on different membranes. It is equally necessary to have some experience of the different types of minor nonspecific lesion which may cause difficulty in interpretation. When working with viruses producing small foci the chief features of the specific lesions are (1) an almost circular form, (2) a central more opaque area of necrosis, (3) a surrounding haze due to inflammatory reaction in the mesodermal layer. As a good working rule, if any two of these features are present the lesion can be taken as specific. The differentiation of primary from secondary foci is sometimes difficult and must be based both on the size and distribution of the lesions. With experience it is usually easy to tell from the general appearance of the membrane whether secondary foci are present or not. Where they are numerous it is probably better to make a rough estimate of the number of primary centres rather than to attempt to assess the significance of each specific focus.

In any large series of counts it will be found that the distribution of counts is asymmetrical owing to the occurrence of a proportion of unduly low counts. It is obvious that with a virus every active particle of which is capable of inducing a lesion under optimal conditions, any deviation of the membrane from normal will tend to lower the count. Abnormally high counts beyond the range of random sampling will only be obtained as a result of technical error or a failure to recognize secondary foci as such. Although a rigid proof is difficult it is our impression that with those viruses which are suitable for work on the chorioallantois the count obtained under optimal conditions is very close to the actual number of virus particles present. Low counts are found particularly with two types of membrane, (1) those with a large nonspecific lesion as a result of haemorrhage during inoculation and (2) membranes easily recognized by the almost complete absence of minor nonspecific blemishes in which foci if present are abnormally small as well as unduly low in number. We have been in the habit of referring to these as nonresponsive membranes.

In evaluating the average count from a group of membranes, usually four, inoculated with the same mixture or dilution we have adopted the convention of dividing membranes into those which could reasonably be regarded as giving counts in the normal range and those which for one of several reasons might give counts well outside the standard range. In calculating the average, counts in the first group are given twice the value of those in the second less satisfactory group. The chief reasons for giving the lower value are large nonspecific lesions, nonresponsive type of membrane and inability to be certain of the extent of secondary focus formation. This weighting of counts seems to be the least objectionable way of utilizing personal experience to assess the relative significance of the counts obtained.

Although the relationship between virus concentration and pock count is approximately linear over the range within which reasonably reliable counts can be made, our experience indicates that there is a consistent deviation from strict proportionality. This deviation is in the same direction as that shown in titrations made by analogous methods of plant viruses such as tobacco mosaic (Youden, Beale and Guthrie, 1935) and of bacteriophages (Dreyer and Cambell Renton, 1933). Figure 1 shows the relation between the average, weighted as described above, of two counts from two decimal dilutions in a series of titrations of vaccinia virus. Counts between three and sixteen foci are indicated by dots on the 45° line, while the counts from the corresponding tenfold concentration of virus are shown by crosses at the appropriate position.

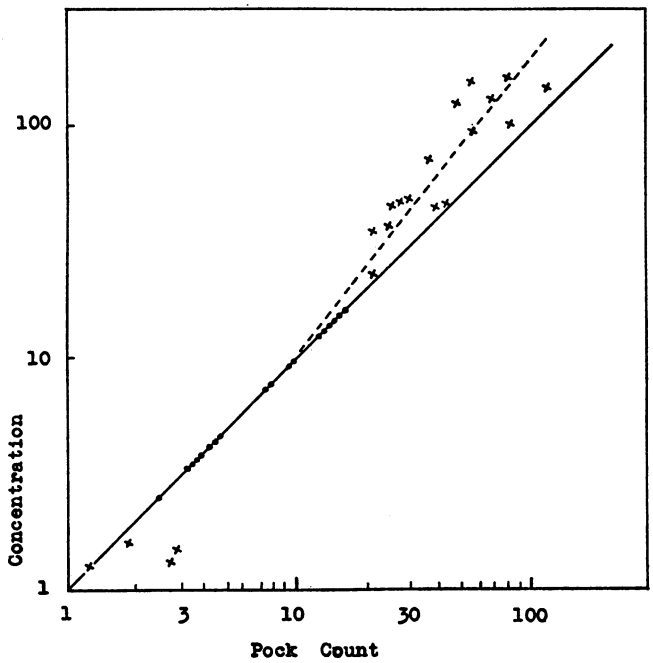

FIG. 1. RELATION BETWEEN VIRUS CONCENTRATION AND POCK COUNT

It will be seen that without exception the counts are lower than would be the case with a strictly linear relationship. For this virus the general nature of the relationship is such as to give a series of the type 1—10—62—300 foci for successive tenfold increases in virus concentration. Wherever possible we prefer to use counts averaging between five and twenty foci for giving a value to the titre of given material. With other viruses the relative diminution in focal counts with increasing concentration is not so evident. With egg-adapted influenza virus strains a simple linear relationship between concentration and count best agrees with the facts.

A detailed titration of a stock of "W.S." egg virus was made primarily to check up the relation between haemorrhage at the time of inoculation and the appearance of the membrane after two days incubation. Dilutions of 1:5000,

1:10,000, 1:20,000, 1:50,000 and 1:100,000 were tested, each on batches of four eggs inoculated through a wide opening to allow a clear view of the initial state of the membrane. Table 1 gives the count recorded for each membrane, the degree of haemorrhage noted at the time of inoculation and the intensity of nonspecific lesions. In this series the averages of the counts fall very close to the values 90—45—22.5—9—4.5 which are proportional to the concentration and there is no indication of any systematic deviation. A glance at the figures

TABLE 1

Titration of influenze virus "W.S. egg" on chorioallantois

A. Relation between virus dilution and focal count

DILUTION	COUNT OF FOCI				WEIGHTED AVERAGE
1:5,000	109	92	88	86	91
1:10,000	50	49	42	40	44
1:20,000	35	25	15	5	23
1:50,000	12	11	11	5	10.5
1:100,000	4	3	2	2	2.75

B. Relation between degree of haemorrhage observed at inoculation and development of nonspecific lesions

DEGREE OF HAEMORRHAGE	NO. OF MEMBRANES	LESIONS AT TWO DAYS							
+	7	++	++	++	+	+	+	+	
±	5	+	±	−	−	−			
−	8	−	−	−	−	−	−	−	−

Under degree of haemorrhage: + signifies easily visible oozing of blood usually giving a patch of at least 1 cm²; ± smaller degrees of visible haemorrhage; − no trace of haemorrhage.

Under nonspecific lesions: +++ (not represented in table) blood stained fluid above membrance with ulcer type nonspecific lesion involving half or more of the inoculated area of the membrane; ++ extensive nonspecific lesion more than 1 sq. cm. in extent; + typical but small nonspecific ulcer; ± minor degrees of nonspecific opacity; − no local traumatic lesions.

will show that the close agreement obtained is better than would normally be expected from the scatter of the counts in each group.

The proportion showing haemorrhage and subsequent nonspecific lesions is unduly high probably on account of the additional manipulation needed with the larger opening in shell and shell membrane. Amongst 22 eggs of the same batch inoculated on the same day by the normal chorioallantoic method one died nonspecifically, two showed +++ lesions, one + lesion and one ± lesions, while the remaining 17 had no nonspecific lesions. There was an absolute correlation between the presence of considerable haemorrhage at the time of inoculation and the appearance of definite nonspecific lesions after incubation.

Preservation of membranes

When it is desired to preserve a membrane as a demonstration specimen it can be mounted in glycerol-gelatin between two lantern slide covers. This method is due to Dr. E. V. Keogh. The membrane is removed from formol

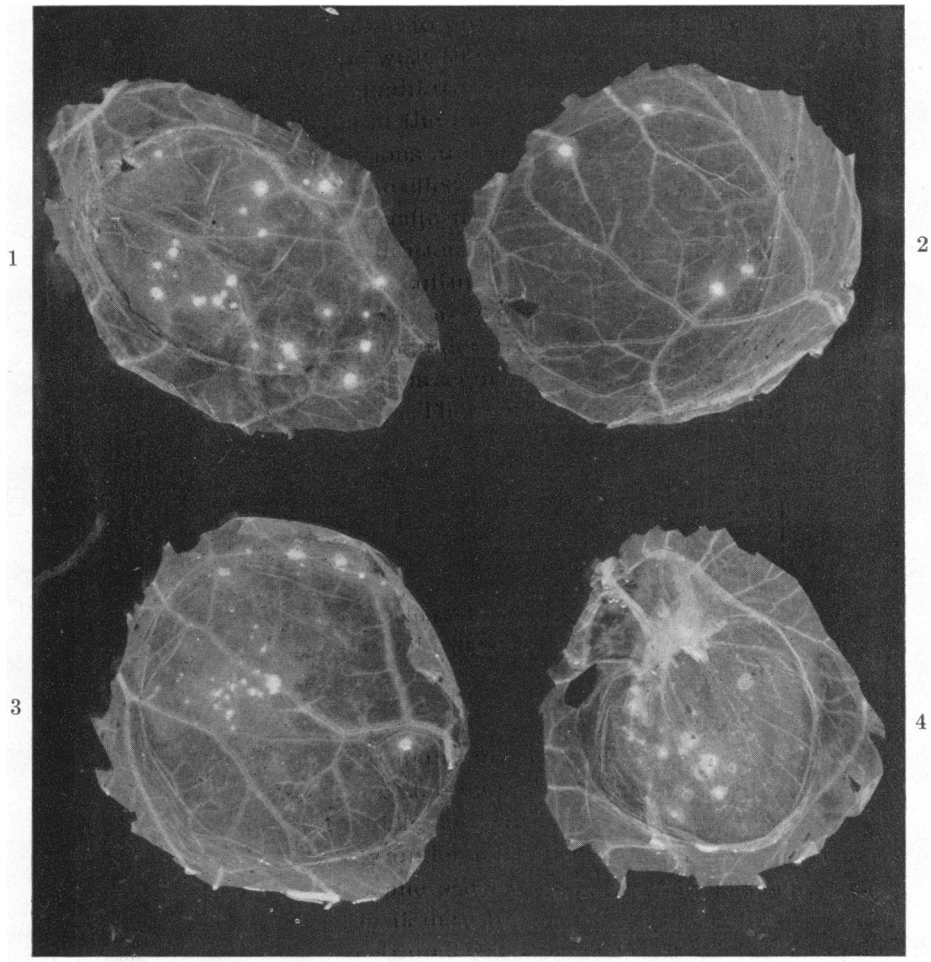

FIG. 2. FOUR MEMBRANES FROM A SERIES OF VACCINIA VIRUS TITRATIONS: NATURAL SIZE
1. Typical satisfactory membrane with 28 specific foci and slight nonspecific oedema; 2. Technically perfect membrane with 4 specific foci; 3. Membrane showing a patch of secondary foci near the centre and some probable secondary foci in association with primary foci at edge. This membrane was interpreted as having 6 primary foci; 4. Typical severe nonspecific lesion resulting from gross haemorrhage at the time of inoculation.

saline and spread carefully on one of the glass sheets, making sure that there are no bubbles of air beneath the membrane. Excess fluid is drained off and 2 or 3 ml. of melted glycerol-gelatin (gelatin 10 gms., water 60 ml., glycerol 70 ml., phenol 0.25 gm.) placed on the centre of the membrane. The upper glass is then lowered on to this and pressed down carefully to avoid trapping air

bubbles. There is sufficient formalin in the membrane to act as a fixative for the gelatin and an antiseptic. The edges of the preparations are bound as in ordinary lantern slides. The mounted membranes are best examined with oblique illumination against a black background but will also give surprisingly clear pictures when projected on to a screen in ordinary lantern slide fashion.

SUMMARY

The technique of quantitative virus titration of the chorioallantois has been modified to avoid as far as possible the nonspecific lesions produced by haemorrhage when the chorioallantois is separated. A drop of sterile saline is used as a fluid wedge to facilitate this separation. A number of other technical points in the processes of inoculating, removing and counting the membranes are discussed.

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