ONE-STEP GROWTH CURVE OF WESTERN EQUINE ENCEPHALOMYELITIS VIRUS ON CHICKEN EMBRYO CELLS GROWN IN VITRO AND ANALYSIS OF VIRUS YIELDS FROM SINGLE CELLS

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A method for assay of animal viruses by plaque count was developed recently (1), based on the property of Western equine encephalomyelitis (WEE) virus to produce necrosis of infected cells derived from the chicken embryo and cultivated *in vitro* as a monolayer of cells on glass. This method was extended to the system formed by poliomyelitis viruses and monkey cells grown *in vitro* (2); in this system plaque production has been used for titration and purification of the virus.

The plaque method has now been applied to a detailed study of the growth characteristics of the WEE virus along lines similar to those followed in bacteriophage work. The present study concerns the one-step growth curve of the virus, both on a cell layer formed *in vitro* and on cells in suspension, and the distribution of virus yields from single infected cells. An outline of the growth cycle of the WEE virus, as inferred from these data, will be given in the discussion.

Both for the WEE and for the poliomyelitis system, a linear relationship between virus concentration and number of plaques has been found. This linear dependence has been discussed and shown to prove that a single virus particle is sufficient to produce a plaque. The number of plaque-forming doses contained in a virus sample is, therefore, directly proportional to the number of the particles it contains. It will be shown in the present article that most of the WEE virus particles, able to produce a plaque, do so under the conditions of our experiments; a plaque-forming dose, can, therefore, be considered approximately equivalent to a plaque-forming particle.

It has been already pointed out in a previous article that the definition of a plaque-forming particle of poliomyelitis virus is an operational one, and that the morphological and genetical properties of such a particle are unknown. The same definition and the same reservations apply to the plaque-forming particle of WEE virus.

Material and Methods

Virus.—A strain of chicken embryo–adapted Western equine encephalomyelitis virus was used throughout the present work. The immediate source of virus was supernatants of infected monolayer chicken embryo cultures, collected 24 hours after infection. The supernatants were stored in a deep-freeze and rapidly thawed before use. They had a titer between 1 and 5×10^9 plaque-forming particles per ml.

Virus Titration.—Virus was assayed by the plaque method. The monolayer tissue cultures were prepared as already described (1). The cultures were grown at 37°C. in unsealed 100 mm. pyrex Petri dishes.[1] The pH of the cultures was controlled by a continuous flow of a 4 per cent CO_2-air mixture in the incubator. A 48 hour culture contained approximately 2.5×10^7 cells, as shown by direct counts.

For plating the virus, tissue cultures of 48 hours were used. The nutrient fluid was sucked away, the cell layer was flooded with 5 ml. of prewarmed phosphate buffered saline (PBS)[2] which was then sucked away; after repeating this washing procedure once more, the cultures were infected with 0.6 ml. of the appropriate virus dilution in PBS. After an adsorption period of 30 minutes at 37°C., the plates were covered with 3.7 ml. of a melted agar overlay of the following composition: 1.3 per cent washed agar in distilled water, 2.4 ml.; fourfold Earle's saline (ES) (3), 0.8 ml.; embryo extract (1:1 in ES), 0.5 ml. (For details of the preparation of the agar-overlay, see reference 2). After 48 hours of incubation at 37°C. under a 4 per cent CO_2-air mixture, the plates were stained with 5 ml. of a 1/10,000 neutral red solution in ES, and the plaques counted after a further incubation of 2 to 4 hours. The plaques stood out as colorless areas in a red background.

Titration of Infected Cells.—In several of the experiments to be reported, in which cells in suspension were infected with the virus, the proportion of cells infected able to release virus had to be determined. This was done by taking advantage of the fact that such infected cells, if plated on a cell layer, can be detected in the same way as free virus particles; each infected cell becomes a center of virus production just as the first cell of the cell layer infected by a free virus particle in the development of a plaque. Each infected cell, therefore, produces a plaque which is indistinguishable from a plaque produced by a free virus particle. In a mixture of infected cells and free virus particles, the proportion of infected cells can be determined by separating the cells from the free virus by one or two cycles of slow speed centrifugation. If non-infected cells are present in the same suspension, they do not interfere with this determination. The number of plaques produced by a washed suspension of infected cells remains a true measure of the proportion of infected cells able to release virus only as long as these cells have not started to release new virus; for this reason, the determination of the number of infected cells should always be done during the latent period following infection (see section on One-Step Growth Curve).

Preparation of Cell Suspensions.—One or more 48 hour cultures were washed twice with PBS, covered with 5 ml. of a 0.025 per cent trypsin solution in ES, and incubated at 37°C. for several minutes until the cell layer became detached from the glass. The cell suspension was transferred to a centrifuge tube and pipetted back and forth several times to break the cell clumps. After centrifugation for 2 minutes at 1000 R.P.M. in an International centrifuge, size 1, and removal of the supernatant, the cells were resuspended in ES. After a second cen-

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[1] The glassware was washed in a 1 per cent solution of "7X detergent," supplied by Linbro Chemical Co., New Haven, Connecticut.

[2] Composition of phosphate buffered saline (PBS): (*A*), NaCl 8.0 gm., KCl 0.2 gm., KH_2PO_4 0.2 gm., Na_2HPO_4 1.15 gm., water 800 ml.; (*B*), $CaCl_2$ 0.1 gm., water 100 ml.; (*C*), $MgCl_2 \cdot 6H_2O$ 0.1 gm., water, 100 ml. Autoclave (*A*), (*B*), and (*C*) separately; mix when cooled.

trifugation, the cells were resuspended in 1 ml. PBS per plate. The suspensions were allowed to stand for 2 minutes to allow larger clumps to sediment. The supernatant was withdrawn, and represented the final cell suspension. It contained from 5×10^6 to 10^7 round single cells per ml.; 10 per cent of the cells, at most, formed clumps of two cells, and larger clumps were exceptional. A distribution of the volume of these cells is given in Fig. 6. Unless specified, experiments involving cell suspensions were carried out in tubes or flasks coated with paraffin to avoid sticking of the cells to the glass.

EXPERIMENTAL RESULTS

The Adsorption of the Virus onto the Cells

To study the rate of adsorption of WEE virus onto a monolayer of chicken embryo cells, parallel cultures were infected with a constant amount of virus, as described under virus titration, and incubated at 37°C. After various time

TABLE I

Adsorption after Different Lengths of Time

All cultures were infected with 0.1 ml. of a stock of Western equine encephalomyelitis virus, diluted $1:5 \times 10^3$. At the time indicated the plates were washed; adsorbed and free virus were determined.

Experiment	Adsorption time	No. of plaques on the adsorption plates	Free virus	Fraction of adsorbed virus
	min.			
1/5/52	5	133/10*	43	0.23
	10	232/10*	29	0.44
	15	27	36	0.43
11/2/52	30	91	16	0.85
	60	136	26	0.84
	90	124	10	0.93
	120	77	14	0.85

* Plates infected with ten times more virus.

intervals, ranging between 5 minutes and 2 hours, the supernatants of a group of cultures were removed, the infected cultures washed, and overlaid with agar. Supernatants and washing fluids were kept. The number of plaques appearing on these cultures gave a measure of the amount of virus adsorbed within the different time intervals. The free virus was measured by assaying the compounded supernatants and washing fluids of the adsorption plates. As seen in Table I, the proportion of adsorbed virus increased rapidly with the time of adsorption until 80 to 90 per cent of the virus was adsorbed. After this time, the increase in adsorbed virus was very small.

The same method could not be used to study adsorption in cell suspensions, containing about 10^7 cells per ml., since no significant decrease of the free virus could be detected after an adsorption period of 30 minutes. The proportion

of virus adsorbed onto suspended cells was therefore measured in an indirect way, based on the determination of the proportion of cells that had become infected after exposure to the virus. This procedure consists of three steps: (1) The proportion of cells infected by different virus concentrations is determined; (2) from the proportion of infected cells, the average number of virus particles adsorbed per cell (called multiplicity of infection) is calculated; (3)

TABLE II

Effect of Different Multiplicity of Infection on the Proportion of Infected Cells

Aliquots of 0.5 ml. of a cell suspension containing 8.2×10^6 cells per ml. were distributed into 6 tubes and infected with different amounts of virus. After an adsorption period of 50 minutes at 37°C., the cells were washed to eliminate the free virus and plated for plaque count at appropriate dilution.

No. of tube	Input: virus particles per ml.	Infected cells per ml. calculated from plaque count	Relative No. of infected cells	Calculated multiplicity	Adsorbed fraction of input virus
1	4.0×10^6	3.8×10^5	0.07	0.07	0.15
2	8.0×10^6	6.4×10^5	0.12	0.13	0.13
3	3.2×10^7	2.6×10^6	0.49	0.67	0.17
4	8.0×10^7	6.4×10^6	}		
5	1.6×10^8	4.0×10^6	} average, 1.0		
6	3.2×10^8	5.6×10^6	}		

the proportion of adsorbed virus is finally deduced from the multiplicity of infection. An experiment of this type, the data of which are given in Table II, will now be considered in detail.

Six tubes containing each 0.5 ml. of the same cell suspension were infected with an input of 0.1 ml. of successive dilutions of a virus suspension of known titer. After 50 minutes of adsorption at 37°C., the various cells suspensions were washed twice by centrifugation at 1000 R.P.M. followed by resuspension of the pellet into 5 ml. ES; this reduced the concentration of the free virus (input virus that had not been adsorbed) to less than $1/1000$ of the initial concentration; *i.e.*, to a level considerably lower than the concentration of the cells. The various cell suspensions were then plated as for virus titration. Since each infected cell able to release virus would produce a plaque under these conditions, and free virus was negligible, the plaque count measured the number of infected cells (for details, see Titration of Infected Cells).

As can be seen from the table, the concentration of infected cells (column 3) increased in proportion to the concentration of the input virus from tubes 1 to 4; no further measurable increase was, however, observed in tubes 5 and 6, which had received the highest virus input. This result shows that only a fraction of the cells had been infected in tubes 1 to 3, which had received smaller inputs, while practically all the susceptible cells had been infected in tubes 4 to 6 with the higher input. (Differences among the tubes 4 to 6 are within the limit of assay fluctuations.)

The ratio between the number of infected cells in a given tube and their maximum number, as observed in tubes with high inputs, gives the proportion of cells infected in that tube. From this proportion, the average number m of adsorbed virus particles per cell (*multiplicity of infection*) can be calculated by assuming a Poisson distribution of virus particles per cell.[3] The use of the Poisson distribution is justified, since it has been proved that one virus particle is sufficient to infect a cell (1, 2). The calculated multiplicity is in each tube proportional to the concentration of the virus added to the tube (compare columns 2 and 5).

The fraction of the input virus adsorbed onto the cells can now be obtained for each tube by dividing the calculated average number of virus particles *adsorbed* per cell—the multiplicity—by the known average number of virus particles *added* per cell. This fraction should be constant in all tubes. As can be seen from the table, the values obtained for tubes 1 to 3 are satisfactorily constant, and show that about 15 per cent of the virus was adsorbed in 50 minutes by a cell suspension of 8.2×10^6 cells per ml.

Some remarks are pertinent to this method of measuring virus adsorption. The highest number of plaques obtained from tubes 4 to 6, measuring the concentration of the virus-releasing cells in the tubes, should approach that determined by counting the cells in a hemocytometer. It was found that the number of virus-releasing cells determined by plaque count was approximately one-half of the total number of cells obtained by direct cell count. A similar discrepancy was repeatedly observed in other experiments. This finding suggests that approximately one-half of the cells in a suspension either do not adsorb the virus, or they adsorb it but do not reproduce it. We cannot distinguish between these two possibilities, but we are inclined to think that the second holds true, chiefly because the cells of the cultures from which the suspensions were obtained seemed to be all similarly susceptible to the infection with WEE virus, being all destroyed by it at a similar time. For this reason, it has been assumed that those cells which did not produce plaques still adsorbed the virus.

Furthermore, the assumption that all cells have an equal adsorbing capacity is very likely not true, due to quantitative and qualitative differences of the surface of the cells. However, these differences do not affect considerably the calculated value of the multiplicity, as inferred from the observed proportionality between multiplicity and input virus (columns 2 and 5 of Table II). In the bacteria-bacteriophage system only very large differences of the surface of the cells affect the calculated value of multiplicity (4).

The One-Step Growth Curve of the Virus

To obtain one-step growth curves, reinfection of susceptible cells by newly released virus must be prevented. Henle *et al.* (5) achieved this result in the case of influenza virus by superinfecting the cells of the allantoic cavity with

[3] Proportion of non-infected cells: e^{-m}.

irradiated virus 1 hour after the infection with active virus. Cairns and Edney (6) achieved the same result by destroying the receptors of the cells of the allantoic sac of the chicken embryo after infection and before the release of new virus by means of the receptor-destroying enzyme from *Vibrio cholerae*. Reinfection can be prevented in cell suspensions by diluting the cells immediately after infection to such an extent that contact of newly released virus with a susceptible cell becomes very improbable. This procedure is commonly used in bacteriophage work (7).

Suspensions of cells in PBS, prepared as described under Methods, containing approximately 5×10^6 cells per ml., were infected with WEE virus; the amount of virus used was such that the multiplicity of infection (average number of adsorbed virus particles per cell) was 4 in some experiments, 0.15 in others. These values were determined in the following way;—The lower multiplicity was measured from the fraction of infected cells in the corresponding tube, as described in the preceding section. The higher multiplicity was calculated from the lower one by multiplying the value obtained for the lower multiplicity by the ratio [concentration of input virus in the tube with higher inoculum]/[concentration of input virus in the tube with lower inoculum]. This could be done since the cell concentration in both tubes was equal.

A volume of 1 ml. of the cell suspensions infected with virus was kept in a 15 mm. test tube at 37°C. for 30 minutes and shaken every 3 to 4 minutes. The cells were then washed by centrifugation as described under Preparation of Cell Suspensions, so as to eliminate the majority of the unadsorbed virus. The cells were resuspended into 1 ml. ES, and then diluted 500-fold into a nutrient mixture of 60 per cent ES, 20 per cent embryo extract, and 20 per cent chicken serum. 10 ml. of this final dilution was pipetted into a 125 ml. Erlenmeyer flask which was then gassed with a mixture of 4 per cent CO_2 in air, stoppered with a rubber stopper, and incubated at 37°C. Samples were taken at various time intervals and plated for plaque count after appropriate dilution. The moment of the dilution of the infected cells into the nutrient mixture was taken as time zero, since virus growth does not take place as long as the cells are in the saline solution, as shown in the following section.

The data from four experiments are given in Fig. 1. As can be seen from the figure, the latent period (time of constant plaque counts) lasted 2½ hours at higher multiplicity and 3½ hours at lower multiplicity. After this time, the plaque counts rose in both cases, first exponentially for 1½ hours, and subsequently more slowly, to reach a maximum 6 to 8 hours later. The curve at high multiplicity remained constantly higher than the other. The plaque counts obtained during the latent period represent the number of virus-producing cells (see under Methods, section on Titration of Infected Cells).

The virus yield (average yield per virus-producing cell) was calculated by dividing the concentration of the virus at a given moment by the concentration of the virus-releasing cells as determined from the platings during the latent period. The maximum average yield of plaque-producing particles varied between 100 and 200.

In some experiments, growth curves were determined in monolayer cultures. Although the occurrence of successive growth cycles could not be prevented

in this case, their effect upon the first part of the growth curve was minimized by infecting the cultures with large virus inocula containing from three to eight times as many virus particles as there were cells in the culture. This assured an initial infection of nearly every cell.

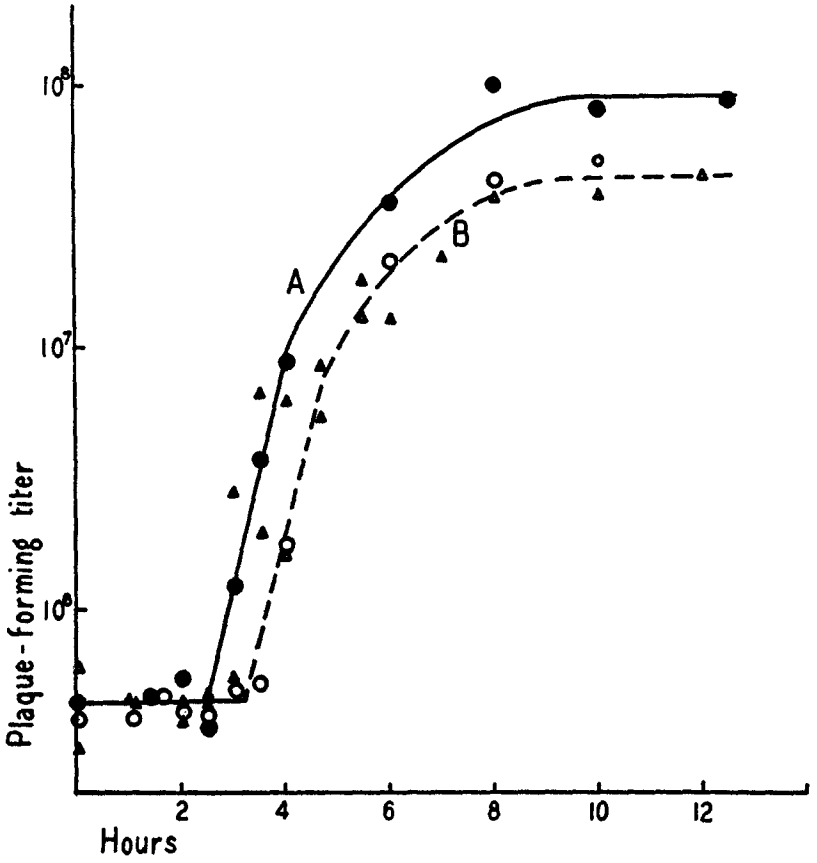

FIG. 1. One-step growth curves of WEE virus on a suspension of chicken embryonic cells. The titers refer to a dilution 1/5 from the adsorption tube. The titers of the low multiplicity experiments were increased by a factor 7 so that the two curves would overlap during the latent period. A, two experiments at a multiplicity 4; B, two experiments at a multiplicity 0.15.

Monolayer cultures were washed with two changes of PBS; after having removed the last amount of washing liquid, the cell layer was infected by covering it with 0.6 ml. of PBS containing from 7×10^7 to 2×10^8 plaque-forming particles. After an adsorption period of 30 minutes at 37°C., the cell layer was washed with two changes of PBS to remove most of the free virus. The cell layers were then covered with 5 ml. of a nutrient mixture, consisting of ES containing 20 per cent of embryo extract. The cell layers were then incubated at 37°C. in an incubator gassed with a 4 per cent CO_2-air mixture. Samples were then plated for plaque

count at various intervals at appropriate dilutions. The moment of the addition of the nutrient fluid was taken as time zero.

The data of one such experiment are plotted in Fig. 2. The initial plaque count represents unremoved free seed virus. The curve shows a small rise

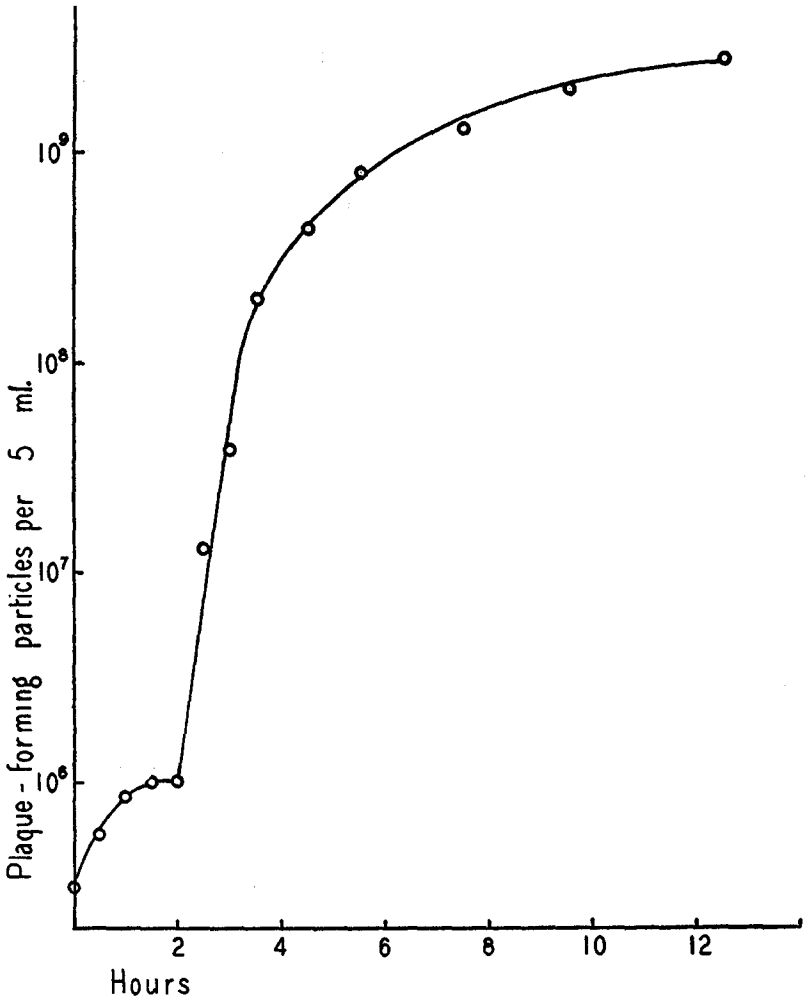

FIG. 2. Growth curve of WEE virus on a monolayer of chicken embryonic cells. The titers refer to the total amount of virus produced in one culture.

during the 1st hour after addition of the nutrient fluid; the titer then remains constant until a new rise begins which continues until after the 12th hour; this second rise is first exponential and later decreases progressively in rate. To assess the cause of the two rises, similar experiments were carried out in

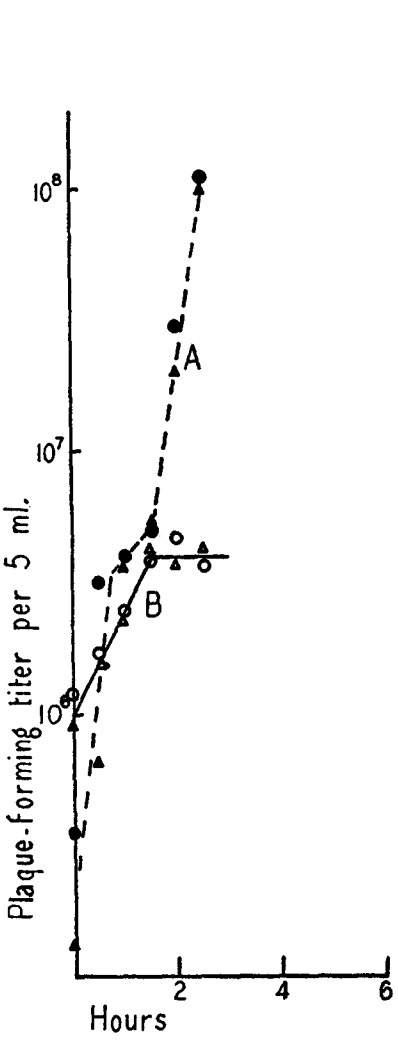

FIG. 3

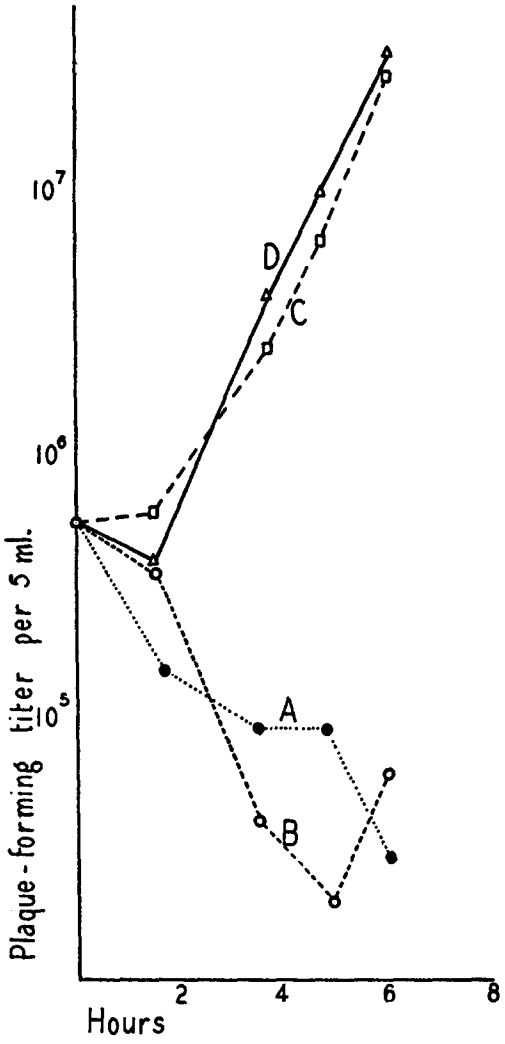

FIG. 4

FIG. 3. First part of the growth curve on a monolayer of chicken fibroblasts. The titers refer to the total amount of virus produced in one culture. A, two experiments in the absence of KCN; B, two experiments in the presence of 5×10^{-3} M KCN.

FIG. 4. Growth curves on a monolayer of chicken embryonic cells in the presence of different fluid mixtures. The titers refer to the total amount of virus produced in one culture. A, Earle's saline without glucose; B, Earle's saline with glucose; C, Earle's saline containing 20 per cent embryo extract; D, Earle's saline containing 20 per cent embryo extract and 20 per cent chicken serum.

which 5×10^{-3} M KCN was added to the nutrient mixture (Fig. 3). In several experiments, it was consistently found that the initial rise took place unchanged under KCN, whereas the second rise was suppressed. This result suggests that the first rise is not due to virus growth but probably to elution of a small fraction of the virus adsorbed, either onto the cells or possibly onto the glass of the Petri dish, whereas the second rise appears to be due to growth of the virus as indicated by the sensitivity to KCN.

In the growth curve obtained from suspended cells, no initial rise was observed. This is very likely due to the small amount of virus involved in this rise. When using a cell suspension, an increase in virus titer will become noticeable only if it is comparable to the concentration of the infected cells (= base line of the curve).

The yield of virus particles per cell on a cell layer was calculated by dividing the total number of virus particles contained in the supernatant of a culture by the number of cells of the same culture, as determined by direct count. The maximum yield per cell varied in various experiments between 200 and 1000, and was, thus, considerably higher than in a cell suspension. This difference seems to indicate a different physiological state of the cells in a monolayer and in a suspension.

Effect on Virus Growth of the Composition of the Medium

To study the effect of different media on virus growth, one-step growth curves were determined, as already described, except that the cells were diluted into media of different composition. The results of such experiments are illustrated in Fig. 4.

As shown in the figure, virus growth was absent in Earle's saline (with or without glucose) while it went on in the presence of embryo extract. Addition of blood serum to the embryo extract did not boost virus production. The decline in plaque count observed in Earle's saline is attributable to such progressive deterioration of the cells that they lose the ability of producing virus when plated.

In contrast to these results, virus growth on the cell layer took place in Earle's saline alone, as proven by obtaining high titer stocks up to 10^9 virus particles per ml. and plaques under this condition. Addition of embryo extract had a favorable effect in that it increased the titer of stocks by a factor of five or more and the number of plaques by a factor of two to three.

Virus growth on the cell layer was unaffected when the pH of the medium was varied between 7.0 and 7.8 by altering the CO_2 concentration in the gas atmosphere of the culture (Fig. 5).

The difference in the growth requirements of the virus, according as it was grown on a cell layer or in a cell suspension, may have been spurious. Since remnants of the culture fluid were removed less efficiently in the case of a cell

layer than in the case of a trypsin-pretreated and washed cell suspension, the experiments might only indicate that extracellular materials are always neces-

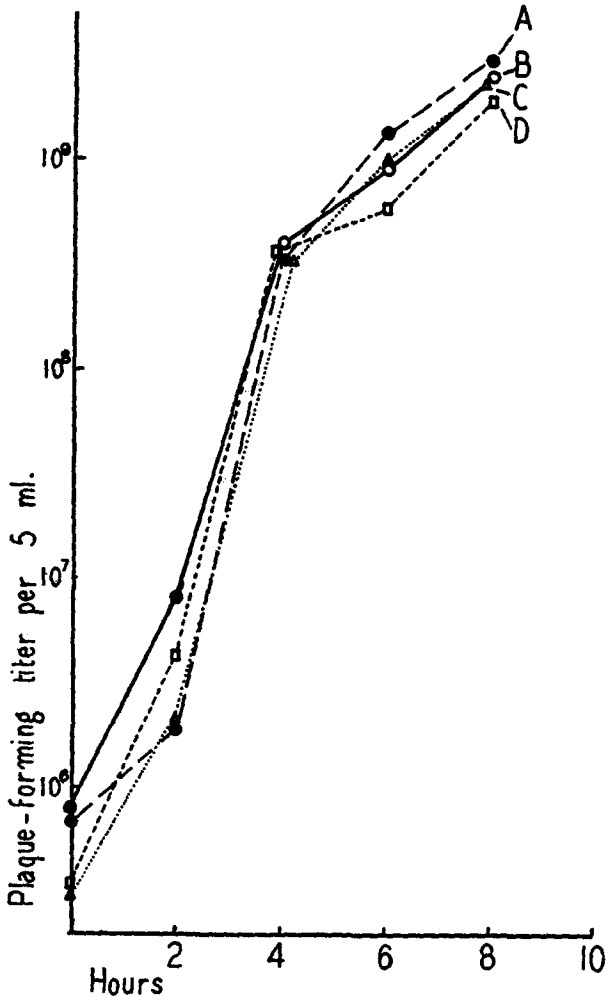

FIG. 5. Growth curves on a monolayer of chicken embryonic cells at different pH of the medium. The pH was controlled by varying the CO_2 tension in the gas atmosphere. The titers refer to the total amount of virus produced in one culture. A, pH 7.8; B, pH 7.4; C, pH 7.2; D, pH 7.0.

sary, either for the growth of the virus or for survival of the cells. This would be in agreement with the findings obtained with bacteriophages. It might be of interest to mention in this connection that Pereira (8) in a study of the growth

of the fowl plague virus in cell suspensions identical with those used here, obtained disproportionately less virus growth when the infected cells, which had been suspended in a balanced salt solution, were highly diluted.

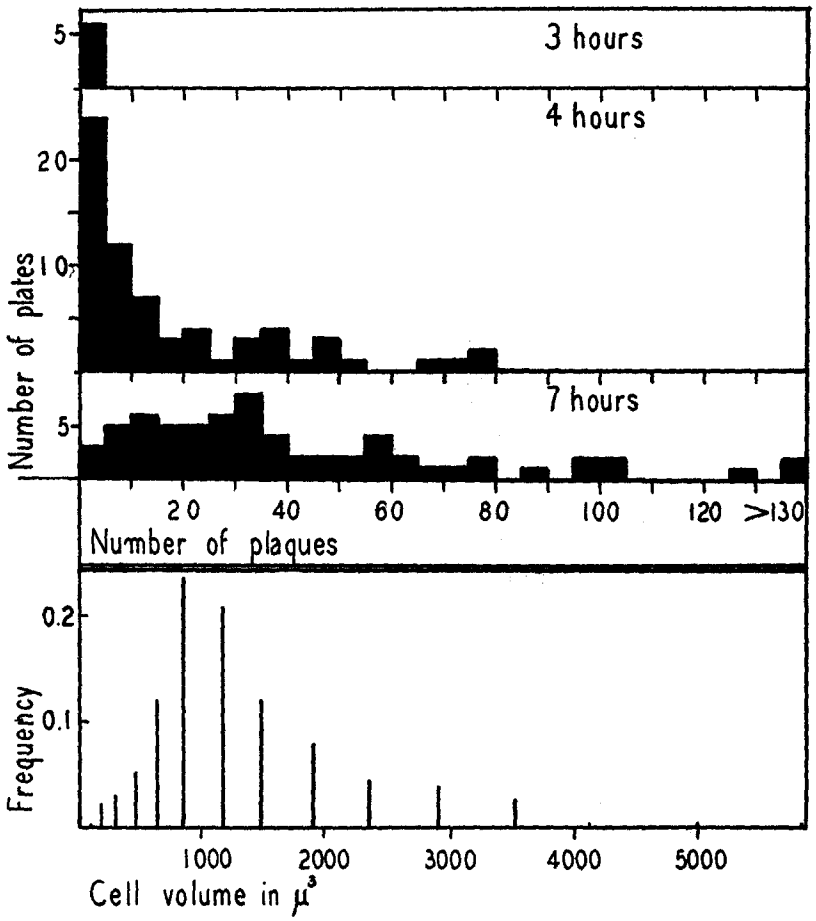

FIG. 6. Distribution of the number of plaques over the productive plates in single cell experiments (3 upper figures) and distribution of cell volumes (lowest figure).

The data for the plaque distributions are pooled from five different experiments; the average number of productive tubes varied in different experiments from 0.5 to 1.1.

The time given with each distribution measures the interval between the dilution of the infected cells into the nutrient mixture and the plating. The data for the distribution of the cell volumes were obtained from microscopic measurements of 382 cells.

These results invite caution in the acceptance of many recent claims that virus growth can be supported by simple or "synthetic" media of various composition, since this effect may be due to small amounts of other materials present in the culture.

Analysis of the Virus Yield of Single Infected Cells (Single Cell Experiment)

This analysis was carried out with the technique used for a similar purpose in bacteriophage work (9).

A suspension of infected cells (multiplicity of infection about 2) was diluted into the usual ES–embryo extract mixture, as described in the section on the one-step growth curve, and distributed into a number of non-paraffin-coated tubes; each tube received 0.5 ml. of the suspension, containing an average of 0.7 virus-yielding cells. As calculated by the Poisson distribution with the parameter 0.7, one-half of the tubes did not receive any infected cell, 70 per cent of the remaining half received one cell, and 30 per cent two or more cells. The tubes were gassed with a mixture of 4 per cent CO_2 in air, closed by rubber stoppers, and incubated. At various times, *i.e.* 3, 4, and 7 hours after diluting the cells with the nutrient fluid, a batch of tubes were chilled to interrupt further virus growth, and the whole content of each tube

TABLE III

Fraction of Cells That Had Released Virus (= Productive Cells) at Various Times during the Rise Period

The fractions are referred to the number of infected cells that formed plaques in platings at time zero.

No. of experiment	Time of plating of the tubes	No. of productive cells in the tubes	No. of plaque formers at time zero	Fraction of productive cells
	hrs.			
207	3	2.4×10^5	1.1×10^6	0.22
208	4	6.8×10^5	2.1×10^6	0.32
209	4	8.0×10^5	1.6×10^6	0.50
214	4	1.5×10^6	2.1×10^6	0.71
206	7	1.5×10^6	1.5×10^6	1.00
214	7	2.2×10^6	2.1×10^6	1.00

plated individually on a plate to determine the number of virus particles. Since the tubes were not coated with paraffin, sticking of the cells to the wall was not prevented and for this reason the infected cells did not appear on the plates, only the newly released virus producing plaques.

A general finding in these experiments was the large variability in the number of virus particles released from different cells, as shown in Fig. 6. It is conceivable that the variability in the cell sizes (lowest distribution in Fig. 6) is partly responsible for this. However, other factors certainly contribute. These will be analyzed in the discussion.

The distributions obtained after various periods of virus growth showed marked differences, both with respect to the fraction of infected cells that had released virus—determined from the fraction of tubes that produced plaques on plating—and with respect to the sizes of the individual yields.

The concentration of productive cells (*i.e.* cells that had released virus at a given time) was compared with the maximum concentration of infected cells capable of producing virus,—determined from platings of the infected cells

during the latent period (compare section on Adsorption). It was found that the ratio of the first to the second value increased almost linearly during the rise period and reached unity after 7 hours (Table III).

The distribution of the sizes of the individual yields after 3 and 4 hours showed that those of small size predominated. The frequency of the small yields decreased considerably in the 7 hour distribution, whereas the number of large yields increased.

DISCUSSION

Experiments on the adsorption of WEE virus onto chicken embryo cells *in vitro* showed that it was much less considerable on suspended cells than on a cell layer. In the experiments reported, 15 per cent of the virus was adsorbed in 50 minutes by a suspension of 8×10^6 cells per ml. with a total surface of 42 cm.^{2}, whereas the same virus fraction was adsorbed within 3.33 minutes onto a cell layer having a surface of 57 cm.^{2} in a volume of 0.5 ml. The probability of adsorption per unit surface, is, therefore, notably greater in the case of a cell layer, as shown by the following considerations:—

The probability that a virus particle will be adsorbed is proportional to the total cell surface per unit volume, to the time, and to a constant characterizing the state of the surface of the cell and of the virus. The product, time $\times$ cell surface, corresponding to a probability of adsorption of 0.15, was found to be 2100 minutes $\times$ cm.^{2} for the cell suspension, and 190 minutes $\times$ cm.^{2} for the cell layer. By taking into account that the volume in the first case was double, we conclude that the adsorption constant was 5.5 times higher for the cell layer.

The conditions which decrease the probability of adsorption in a cell suspension may be a consequence of the method of preparation of the suspension. The use of trypsin—which is known to have a depressing effect on the adsorption in other virus cell systems (10)—might be an important factor.

The growth curves of the WEE virus obtained both on a monolayer tissue culture and in a suspension of cells, showed a latent period varying from 2 to 3½ hours, which was followed by a steep increase in virus production lasting over several hours. The latent period varied with the conditions of the experiment and with the multiplicity of infection. The latent period was longer in cell suspensions; this may be partly owing to the time required for the virus titer to become comparable to the concentration of the infected cells; for this reason the shorter latent period determined on the cell layer represents very likely the real latent period. The latent period was shorter at high multiplicity of infection, as previously found by Liu and Henle (11) in the case of influenza virus B. This difference again may be apparent and due to a steeper rise of the curve obtained at high multiplicity, with result that the virus titer sooner reaches in this case the titer of the infected cells. The persisting difference in

the height of the two curves during the rise periods at high and low multiplicity is, however, real; it probably indicates that more than one virus particle can participate in the intracellular growth of this virus, a phenomenon known to occur in bacteriophages (12).

The growth curves and the analysis of the amount of virus released by single cells supply information on several aspects of the growth of the virus.

A constant characteristic of the growth curves, both in a cell suspension and on a cell layer, was the exponentiality of the first part of the rise period. The question arises whether this exponentiality of the curve reflects the exponentiality of some phase of the intracellular virus growth. The following analysis of the single cell experiments will clarify this point.

We find, in the first place, that the increase in virus titer during the rise period is due to the concurrence of two factors: a linear increase with time of the number of cells releasing virus, and a concomitant increase in the number of virus particles released by each cell.

In the second place, it can be seen that individual infected cells release virus over a long period of time. This conclusion is derived from a comparison of the virus content of individual tubes at various times during the rise period, which shows a striking difference in the proportion of tubes containing 10 or less virus particles (called low yielders) at 4 and 7 hours (60 per cent against 11 per cent, respectively). This difference is partly due to the appearance, between the 4th and 7th hour, of new productive cells in tubes generally containing large yields; as a consequence of which, even if the absolute number of tubes containing low yields remained constant during this period, their proportion would decrease. It can be shown,[4] however, that this factor

[4] To determine with accuracy the fate of the low yielders present at 4 hours, we must calculate from our data the fraction of low-yielding *cells* at the 4th and 7th hour. This fraction is actually different from the fraction of *tubes* producing 10 or less plaques on plating, because of the presence of tubes containing more than one cell.

A tube has a low yield when it receives a single low-yielding cell and occasionally when it receives more than one low-yielding cell. We cannot estimate precisely how many tubes of the latter class have a low yield. We will assume that all of them do and therefore that all tubes containing one or two low-yielding cells and no high-yielding cells are themselves low-yielding.

Since the distribution of cells per tube is Poissonian, the proportion of low-yielding tubes among all the tubes in a batch can be calculated to be equal to

$$\left(an + \frac{(an)^2}{2}\right) e^{-an}\, e^{-(1-a)n} = L$$

where a = proportion of low-yielding cells among all the cells that have yielded virus at the moment of plating; n = average number of virus-yielding cells per each tube in a given batch.

In this equation L can be experimentally determined, n can be calculated from the fraction of sterile tubes in a given batch; a is the unknown amount, to be calculated.

For this calculation we use data obtained from a single experiment, in which a batch of tubes was plated at 4 hours, another at 7 hours. We first calculate n in the 4 hour and 7 hour batch, respectively: 4 hour batch: 60 tubes, 20 of which sterile (no plaques produced). n =

accounts only for a small part of the decrease in the proportion of small yields and that most of the small yields present at 4 hours have actually disappeared at 7 hours. This disappearance must have been brought about by new release of virus between the 4th and 7th hour by cells which had already released virus before the 4th hour.

It cannot be decided from the experiments whether virus particles are released individually or in groups. However, the fact that many plates with only few plaques were found in the 4 hour platings suggests that virus is released either in very small parcels or continuously. Thus the release of WEE virus differs from the sudden release ("burst") of bacteriophage by lysis of the bacterial cell. A second animal virus for which a gradual release of virus has been made very probable is the influenza virus (13, 14). The gradual release may therefore be characteristic of many animal viruses.

In view of the information of various kinds supplied by the single cell experiments, the exponential increase of the growth curve is interpreted most simply by assuming that the amount of complete virus released by single cells increases exponentially with time. A simple mathematical theory supporting this statement will be presented elsewhere.

If this interpretation is correct, the exponential release may reflect a stage of exponential multiplication during the intracellular growth of the virus, provided other processes involved in the intracellular growth are not limiting. This finding is in agreement with findings of different nature obtained in bacteriophages and supporting the same conclusion (15).

SUMMARY

The rate of adsorption of WEE virus onto chicken embryo cells *in vitro* was determined both on a cell layer and on a cell suspension.

One-step growth curves were determined in cell suspensions and on cell layers. The latent period varied between 2 and 3½ hours; it was shorter on cell layers and decreased with higher multiplicity of infection. The shortest period is probably the real latent period.

The growth curves of the virus showed an initial exponential rise and reached a maximal constant value after 6 to 8 hours. The maximum virus yield per

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$e^{-20/60} = 1.1$. 7 hour batch: 75 tubes, 34 of which without plaques. $n = e^{-34/75} = 0.79$. The value of n was different in the two batches due to a different dilution used and a different proportion of yielders. The quantity L was $19/60 = 0.317$ in the 4 hour batch, and $4/75 = 0.053$ in the 7 hour batch.

From these data we calculate $a = 0.58$ at 4 hours, $a = 0.13$ at 7 hours.

In this experiment, 69 per cent of the cells had yielded virus at 4 hours and 100 per cent at 7 hours; if the new yields had been all large yields, a should decrease from 0.58 to 0.40 at 7 hours. The observed value is, on the contrary, only ⅓ of it. We conclude that at least ⅔ of the low-yielding cells present at 4 hours have passed into the class of high yielders at 7 hours.

cell varied between 200 and 1000 on the cell layer, and between 100 and 200 in suspended cells.

The yield of single infected cells was determined. An analysis of the distributions of the individual yields obtained after various periods of virus growth led to two main conclusions: (1) that virus is released from the same cell over a long period of time; (2) that one phase of the intracellular virus growth is exponential.

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