THE GROWTH *IN VITRO* OF VACCINIA VIRUS IN CHICK EMBRYO CHORIO-ALLANTOIC MEMBRANE, MINCED EMBRYO AND CELL SUSPENSIONS

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(With 8 Figures in the Text)

The behaviour of vaccinia virus after its inoculation on the chorio-allantoic membrane of the developing chick embryo was described in detail by Maitland & Tobin (1956). In assessing the total amount of virus that could be recovered it was necessary to titrate the liquid, which, during the process of harvesting the membrane, collected in the shell when the membrane was detached and removed, as well as to titrate virus in the membrane itself. Immediately after inoculation the amount of virus recovered from both sources, estimated by pock-counts, was greater than the amount inoculated (termed enhancement of infectivity). At first most of the virus was in the liquid, but during incubation of the egg it gradually passed from the liquid to the membrane. As a consequence the amount of virus in the membrane rose for a time, but concurrently some loss of virus occurred so that the amount in the membrane itself at a particular time depended upon a balance between accumulation and loss; the amount rose for about an hour and then began to fall. The total amount of virus however decreased gradually from the start and reached a minimum in 2–3 hr. being then 20 % or more of the amount detected immediately after inoculation (i.e. allowing for initial enhancement). Increase of virus due to growth was detected 4–5 hr. after inoculation.

Anderson (1954) noted a gradual loss of infectivity of vaccinia virus, after inoculation on the chorio-allantoic membrane; during the first 9 hr. about 90 % disappeared. At 10 hr. the titre had begun to rise and it increased steadily from then onwards. Additional observations led to the conclusion that the majority of the inoculated virus entered an 'eclipse phase' during the first 9 hr., the implication being that it went through a non-infective stage as part of a cycle of multiplication. This, however, was an assumption for there was no conclusive evidence to indicate whether the newly formed virus was derived from that part of the inoculum which became non-infective or from the part which retained its infectivity. Either was possible. Briody & Stannard (1951) using egg-passaged virus noted a very marked drop in infectivity of virus during the first hour after inoculation on the chorio-allantoic membrane, a latent period up to 8 hr., with a sudden large increase of virus at that time and another steep rise in titre at about 16 hr. These findings were interpreted as indicating that a major change in the virus particle, masking its infectivity, was part of a growth cycle. Again, this was an assumption and no further experiments were made in support of it.

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The shape of the curve of changes in titre reported by Anderson differed from that of Briody & Stannard in showing a gradual preliminary fall during 9 hr. instead of a steep fall for 1 hr. and in showing a steady and continuous increase instead of step-wise increments.

Crawford & Sanders (1952) cultured the virus in thin pieces of skin, removed from a rabbit's ear after intradermal inoculation, by incubating them in a nutrient medium. There was a decline in infectivity during the first few hours of incubation and a rise in titre after about 10 hr.

In order to avoid the complexity of the conditions *in vivo*, experiments to be reported here were made *in vitro*, using chorio-allantoic membranes, minced chick-embryo or cell suspensions, which enabled contact between tissue or cells and virus to be controlled and ensured that changes in infectivity of virus were due to processes taking place in or on the tissue or cells.

MATERIALS AND METHODS

The strain of virus was that used at the Lister Institute for preparing small pox vaccine. It was repeatedly passed in the skin of rabbits. Elementary body suspensions were prepared from infected rabbit skin by the method of Hoagland, Smadel & Rivers (1940); they were stored at -20 to $-40°$ C. and thawed only once, immediately before use. Virus was titrated by inoculating 0·05 ml. of ten-fold dilutions on the chorio-allantoic membrane of eggs incubated for 12–13 days. Eight eggs were used for each dilution. They were incubated further at 37° C. for 2 days when pock counts were made. The infectivity was expressed as the number of pocks (infective units of virus) per ml.

GROWTH-CURVE EXPERIMENTS
I. Chorio-allantoic membranes
Experimental procedures

Chorio-allantoic membranes were removed from eggs that had been incubated for 12–13 days by detaching the membrane from a piece of shell which had been cut around and separated from the egg (as in harvesting membranes for pock-counting). Each membrane was put into 1 ml. of a known amount of virus and kept at 37° C. for 10 min. with frequent gentle agitation by hand. The membranes were removed and washed in pairs in 10 ml. of Hanks' solution in a 25×150 mm. test tube which was held against an eccentric rapidly rotating rubber bung so that the bottom of the tube made about 400 double excursions of about 3 cm. amplitude during 1 min. The membranes swirled around rapidly in the tube during this time. They were transferred to a similar tube and the washing repeated. Some membranes were then ground and titrated as a base line of infectivity. Others were put in pairs into a shallow layer of Hanks' solution (4 ml. in a 50 ml. conical flask) and incubated at 37° C. in air, to be ground and titrated at intervals. The experiments were arranged so that the product of four similar membranes was pooled for each titration; the membranes were disintegrated in a Griffiths' tube as

described by Maitland & Tobin (1956). Tissue suspensions and dilutions of all materials for titration were made in Hanks' solution.

Results

Enhancement of infectivity of virus occurred when a membrane was put into a suspensions of virus in order to infect it. The amount of virus in the membrane after contact plus the amount left in the suspension was usually greater than the original amount of virus. This, however, was incidental to the present experiments and did not affect the validity of the base line titration.

The amount of virus in membranes after they had been in contact with virus and drained of excess of virus, but before they were washed, ranged between 20 and 40 % of the original titre of virus in the tube. After washing, which was intended to remove virus loosely adherent to the membrane in a surface film of liquid, and to leave only virus which was firmly attached to the tissue, the amount of virus on the membrane was usually 5–10 % of the virus originally in the suspension.

A similar result was obtained if contact was at room temperature, about 18° C. Contact for longer periods up to 60 min. did not result in appreciably greater adsorption. The period of contact for 10 min. was thus adequate and, in determining a base line of infectivity, was preferable to a longer period because it avoided the possibility of longer contact resulting in loss of infectivity of the virus in the membrane. The base line in these experiments thus represented virus firmly attached to the tissue and it was determined before any appreciable loss of infectivity was likely to have occurred.

The changes in titre of virus in the membrane during incubation *in vitro* were of the same kind as occurred *in vivo* but they took place more slowly.

The data for three typical experiments are shown in Table 1. By titrating at relatively short intervals, the trend of change in titre became evident although a few individual figures were out of line. The curves of change in titre are shown in Fig. 1. There was gradual loss of infectivity for approximately 10 hr. At the lowest point the virus that remained was 25 % or more of the amount present in the membrane at the start. Increase was clearly detectable at 18–20 hr., and began earlier in some experiments. No clear evidence for step-wise increases was obtained but the experiments were not designed to study this point. Similarly, it was not possible to deduce the exact time at which multiplication of virus began in individual cells. Although all the cells that were originally infected must have become infected within a period of about 10 min. the speed of events taking place within the cells may not have been uniform. Further, the change from a falling to a rising titre depends on the balance between continuing loss and start of growth of virus in the membrane as a whole.

These results agree with those obtained *in vivo* (Maitland & Tobin, 1956) in demonstrating a similar loss of infectivity prior to multiplication and in confirming that this loss occurred in the membrane. In both sets of experiments at least 20–25 % of virus, sometimes more, remained infective. The lowest point of infectivity and the time when virus began to increase occurred later *in vitro* than *in vivo*.

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Table 1. *Changes in titre* of virus in chorio-allantoic membranes incubated in Hanks' solution*

Hours of	Experiment		
incubation	39	40	42
0	100	100	100
	(29,000)†	(28,800)†	(31,800)†
2	—	—	88
4	—	77	106
6	—	—	67
8	26	82	—
10	35	24	—
12	46	33	29
14	120	42	35
16	107	24	55
18	386	70	27
20	414	84	540
22	—	—	143
24	770	560	—
30	725	810	396

* Expressed as percentage of the titre at 0 hr.
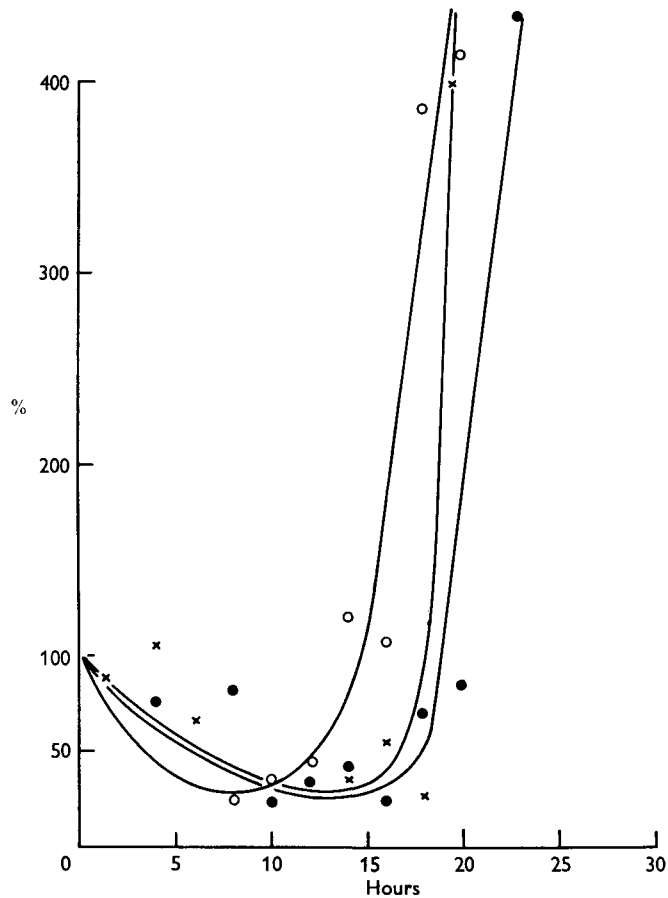† Number of infective units of virus (pocks) per membrane at 0 hr.

Fig. 1. Growth curves of vaccinia virus in chorio-allantoic membranes incubated in Hanks' solution.

The virus remained almost entirely in the membrane. In one experiment after incubation for 6 hr., 3 % of the total virus in the flask was in the medium, and after 20 hr. only 0·3 %.

II. Heated and disintegrated chorio-allantoic membranes
Experimental procedures

In order to obtain further evidence which might help to decide whether the initial fall in titre of virus in membrane was or was not an essential part of virus reproduction, experiments were made, similar to those described in the previous section, but using heated and disintegrated membranes in which the virus would not grow.

Heated membranes. Membranes were immersed in Hanks' solution and heated in a water-bath before being put in contact with virus. Exposure to 45° C. for 20 min. followed by contact with virus, washing and incubation, as in the previous experiments, did not prevent growth of virus which occurred in less than 20 hr. Heating at 45° C. for 40 min. also failed to abolish the growth of virus, but it began later and was detectable between 24 and 48 hr. When membranes were heated at 50° C. for 20 min. there was no growth of virus; after 48 hr. incubation the titre of the virus was declining. This amount of heating was therefore adopted as the minimum that would abolish growth of virus and in what follows this is connoted by the term 'heated membranes'.

Heated membranes were exposed to virus, washed, incubated and titrated as described for normal membranes.

Disintegrated membranes. Normal membranes were put in contact with virus as in the growth-curve experiments. They were then washed, as described, placed in chilled Hanks' solution (2 ml. per membrane) and disintegrated in an 'ato-mix' blender * running at full speed for $2\frac{1}{2}$ min. From fourteen to sixteen membranes were disintegrated at one time; the Hanks' solution and the head of the machine were chilled to avoid producing a temperature which might harm the virus during the operation. The temperature did not rise above 21° C. There was almost total disruption of cells; at most only a few intact cells could be seen. The suspension of disintegrated infected tissue was incubated in 50 ml. conical flasks, 4 ml. per flask, the proportion of tissue and medium being the same as with intact membranes; the equivalent of four membranes was pooled and titrated at intervals.

Heated and disintegrated membranes. Heated membranes were exposed to virus, washed, disintegrated, incubated and titrated as described for the previous experiments.

Results

Heated membranes absorbed as much virus as normal membranes so that the base lines of infectivity, after washing and before incubation, were quantitatively comparable. During the first 10 hr. of incubation the fall in titre of virus in heated membranes was similar, both in rate and amount, to the fall which preceded

* Made by Measuring and Scientific Equipment Ltd., London.

growth of virus in normal membranes, but, instead of the subsequent rise in titre due to growth, the virus in heated membranes continued to decrease. With disintegrated membranes the rate of fall in 10 hr. was somewhat less and with membranes which were both heated and disintegrated it was intermediate. After about 10 hr. the rate of fall in all the experiments became much slower; a considerable amount of virus remained at 48 hr. and in experiment 71, the only one tested after longer incubation, virus was found after 6 days (Table 2 and Fig. 2).

Table 2. *Changes in titre* of virus in heated or disintegrated chorio-allantoic membranes incubated in Hanks' solution*

Hours of incubation	Heated 50° C. 20 min. Experiment		Disintegrated. Experiment		Heated and disintegrated. Experiment	
	66	69	70	71	73	75
0	100 (23,500)†	100 (36,400)†	100 (61,700)†	100 (4,800)†	100 (8,500)†	100 (32,980)†
1	117	30	88	92	85	86
3	52	71	78	72	73	39
5	10	27	67	59	57	29
9	—	—	—	—	51	37
10	13	30	69	25	—	—
24	12	44	45	61	60	58
48	12	6	47	40	31	28
6 days	—	—	—	4	—	—

* Expressed as percentage of the titre at 0 hr.
† Number of infective units of virus (pocks) per membrane at 0 hr.
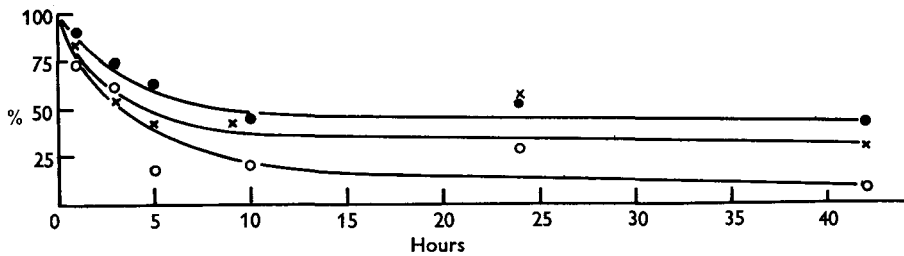

Fig. 2. Changes in infectivity of vaccinia virus incubated in heated and disintegrated membranes. Each curve represents the average of two experiments. ○, heated; ●, disintegrated; ×, heated and disintegrated.

III. Minced chick-embryo

Embryos, 12 days' old, less the head and legs, were finely minced with scissors. The mince was washed successively in two lots of 10 ml. Hanks' solution per embryo; after mixing it was allowed to settle by gravity. The washed tissue was infected by adding to 1 vol. mince, 1 vol. virus and 3 vol. Hanks' solution, and keeping this at room temperature for 10 min. with gentle agitation by hand. The mince was then allowed to settle and washed twice, each in 5 vol. of Hanks' solution, by mixing and settling under gravity. One volume of infected and

washed mince was mixed with 19 vol. Hanks' solution, distributed in 5 ml. amounts to $2\frac{1}{2}$ in. Petri dishes and incubated in air at 37° C. At intervals the tissue was ground in a Griffiths' tube, mixed with the liquid part of the culture, and titrated.

The results of two typical experiments are shown in Table 3. In general they corresponded with those obtained for chorio-allantoic membranes; there was a marked fall in titre for about 10 hr. to 30–40 % of the original titre, and in 24–26 hr. a clear indication that growth had occurred.

Table 3. *Changes in titre* of virus in minced chick-embryo incubated in Hanks' solution*

Hours of incubation	Experiment	
	129	133
0	100	100
	(24,000)†	(3920)†
2	73	67
4	57	35
6	40	77
8	35	29
10	47	43
12	—	92
24	63	290
26	254	510
28	350	780
30	527	1350
48	2900	14600

* Expressed as percentage of the titre at 0 hr.
† Number of infective units of virus (pocks) per ml.

IV. Chick-embryo cell suspensions

Experimental procedures

Twelve-day-old embryos less head and legs were minced and washed twice in 4 ml. Hanks' solution per embryo by mixing and allowing the tissue to settle. To the deposit, 8 ml. per embryo of 0·25 % Difco trypsin in phosphate-buffered saline (Dulbecco & Vogt, 1954) was added and allowed to act for 10 min. at 37° C. with agitation at intervals. The suspension of cells thus obtained was filtered through a 100 mesh per inch gauze. The cells were then deposited in a horizontal centrifuge at 1000 r.p.m. for 5 min., washed by similar deposition three times from equivalent volumes of Hanks' solution, and finally suspended in Hanks' solution to give 10×10^6 cells per ml. To this 1/20 volume of a suitable dilution of virus in Hanks' solution was added. Contact was for 10 min. at 37° C. The cells were again deposited and the supernatant fluid removed. The deposit was resuspended in culture medium to contain 2×10^6 cells per ml. and distributed in 6 ml. amounts to $2\frac{1}{2}$ in. Petri dishes which were incubated in air at 37° C. The medium unless otherwise specified was 20 % chick-embryo extract and 0·5 % casein hydrolysate (enzymatic) in Hanks' solution.

For titration the supernatant fluid was removed from a Petri dish and 2 ml. of 0·25 % Difco trypsin in phosphate-buffered saline added and allowed to act for 10 min. at 37° C. This was required to detach the cells from the glass, particularly when they had stuck after a few hours of incubation. (As the trypsin had the effect of increasing somewhat the titre of the virus (see Maitland & Tobin, 1956), it was allowed to act similarly on the controls which were samples of the bulk culture, as distributed into Petri dishes before incubation). The trypsin plus detached cells was added to the supernatant fluid and the dish washed out with 4 ml. Hanks' solution plus a volume equal to any loss of fluid during incubation of the culture. The cells from this 12 ml. of material were deposited in an angle centrifuge and the supernatant fluid put on one side. The deposit was resuspended in 12 ml. Hanks' solution and treated in an M.S.E. 'masticator' for 5 min. to break up the cells. The material was in a 25 ml. universal container and the machine was run at full speed; over 95 % of the cells were disintegrated. The treatment had no effect on the titre of virus in Hanks' solution. The cells were masticated in Hanks' solution to avoid excessive frothing which occurred with the culture medium. The masticated cells were then added to the supernatant fluid from the last centrifuging, making a volume of 24 ml. which was titrated.

Freshly prepared cells were used for most experiments, but cells kept overnight at 4° C. were found to be satisfactory. During preparation sometimes a small amount of clumping occurred, but the clumps could be readily dispersed or removed by refiltering through the metal gauze; the suspensions used for the experiments were practically free from clumps, at most only an occasional small microscopic clump was present. In the Petri dish the cells settled, and many stuck to the glass, spread out and multiplied if the cultures were incubated long enough, but only a small proportion (about 1 %) of the cells was infected at the beginning of the experiment. The cells in the suspension were obviously not all of the same type, as was to be expected, nor were they uniform as regards their physiological intactness as many cells, although morphologically apparently healthy, failed to stick to the glass and became rounded and granular. The number of infected cells could be determined by titrating the cell suspension unmasticated and thus the percentage of cells originally infected was estimated.

In order to avoid as far as possible difficulties in interpreting the results of titration owing to variability inherent in the technique three precautions were taken; (a) titrations at frequent intervals were made so that a trend would be apparent even if an occasional reading was out of line; (b) two or even three controls were put up and titrated separately and the average taken as a base line; (c) for each reading duplicate cultures were titrated separately and the average taken.

Results

In the system of cells and medium used for these experiments the fall in titre of virus which preceded growth was relatively small, and in this respect differed from the behaviour of the virus in chorio-allantoic membranes and minced embryo. The data for representative experiments are in Table 4 and Figs. 3–8. In a number

of experiments the level of virus remained at 85–90 % of the original titre until growth began and in the others it seldom fell below 65–70 %; only in an occasional experiment did the titre drop to 40–50 %.

Table 4. *Changes in titre* of virus in chick-embryo cell suspensions incubated in nutrient medium*

Hours of incubation	Experiment				
	147	148‡	149§	150§	153‖
0	100	100	100	100	100
	(19,600)†	(8,400)†	(23,700)†	(6,600)†	(24,500)†
1	68	90	106	70	84
2	73	83	90	82	76
3	76	56	83	115	77
4	74	95	118	65	98
5	77	58	112	57	97
6	55	40	108	81	98
7	72	55	99	78	88
8	70	73	83	104	104
9	70	53	68	69	—
10	—	—	83	—	—
11	—	—	123	60	—
12	91	—	—	—	—
14	82	—	—	—	—
16	98	—	—	—	—
22	134	—	—	—	—
23	—	220	276	260	—
24	168	—	—	—	—
25	—	366	320	266	—
28	338	585	450	—	—
30	415	730	480	1080	—
48	5700	—	—	—	> 3000

* Expressed as a percentage of the titre at 0 hr.
† Number of pocks per ml. at 0 hr.
‡ The figure at 0 hr. is the average of 2 control titrations.
§ The figure at 0 hr. is the average of 3 titrations.
‖ The figures throughout are the average of duplicate titrations.

The fall in titre that did occur was gradual, the period of lowest values occurring between 5 and 10 hr. The infective titre of the cultures usually began to increase between 15 and 20 hr., which was confirmed by other titrations made especially to cover the period between 16 and 24 hr., and at 24–45 hr. it was rapidly rising.

Observations made specially to detect any rapid or extensive loss of virus during the first 90 min. following contact between virus and cells failed to reveal any such occurrence. Thus the control titrations represented a proper base line as no rapid loss of virus occurred during the manipulations required to prepare the controls for titration.

The medium in which the cells and virus were incubated affected the outcome. In two experiments in which infected cells were incubated in Hanks' solution the virus declined to about 30 % of the original amount in 5–10 hr. and to 10–15 % in 24 hr. In two experiments 20 % chick-embryo extract in Hanks' solution was

used; in one the level of virus remained at about 90 % of the original amount and in the other at 75–80 % during the preliminary period; in both experiments growth began somewhat later than when the chick embryo cells were present in the culture medium.
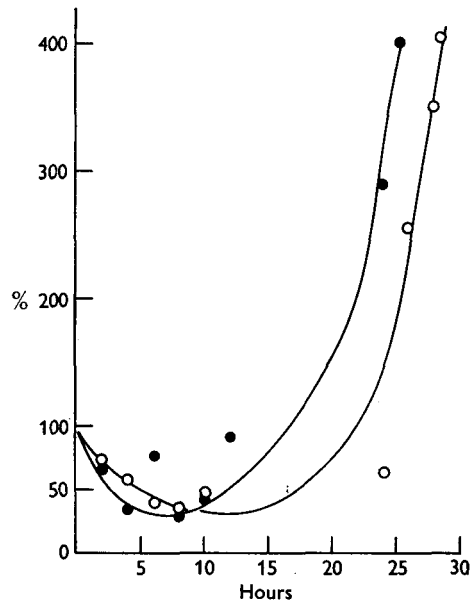
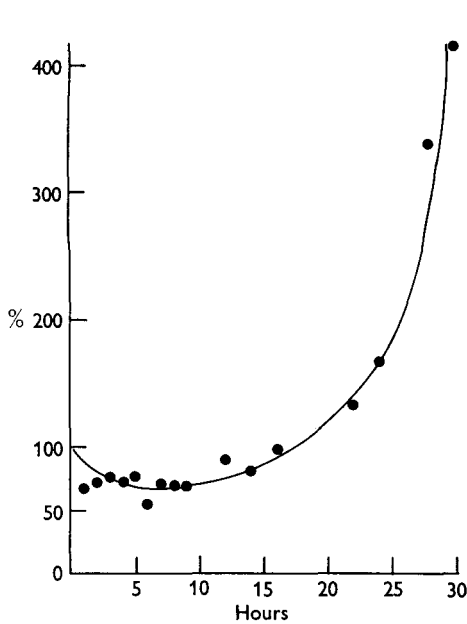Fig. 3. Growth of vaccinia virus in minced chick-embryo incubated in Hanks' solution.
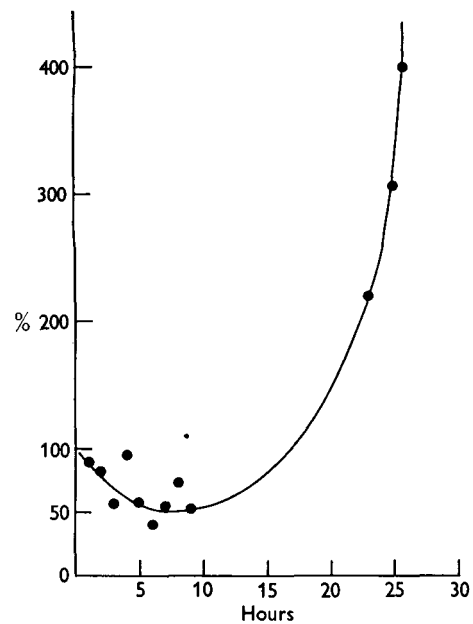
Fig. 4. Experiment 147.

Fig. 5. Experiment 148.

Figs. 4–8. Growth curve of vaccinia virus in chick-embryo cell suspension incubated in a nutrient medium.
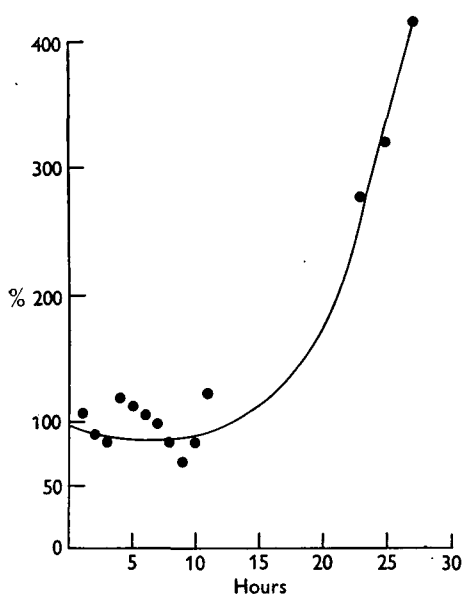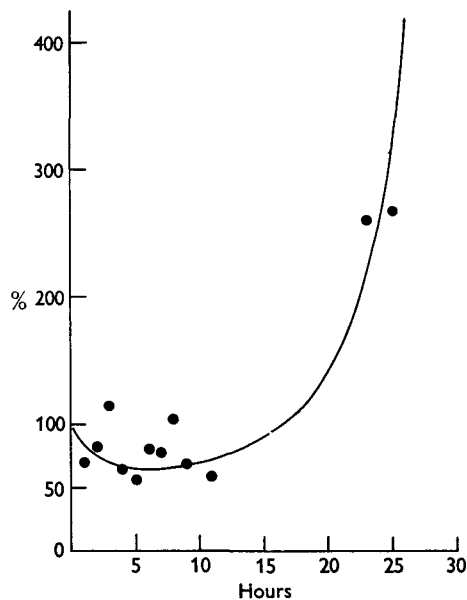
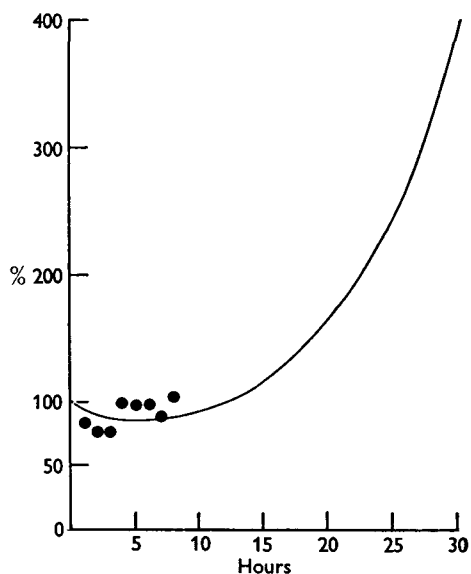Fig. 6. Experiment 149.

Fig. 7. Experiment 150.

Fig. 8. Experiment 153.

DISCUSSION

An endeavour had been made in these experiments to obtain evidence for or against the view that the fall in titre of vaccinia virus in infected tissue which precedes multiplication of virus indicates a so-called 'eclipse phase', that is, a transformation of virus into a non-infective form in which state it is reproduced to be changed by a further process into mature and infective virus. The experiments have shown that the fall which occurs in the chorio-allantoic membrane *in vivo* was

paralleled when infected membrane or minced chick-embryo was incubated in culture. This in itself is not sufficient to warrant the assumption that the loss of infectivity is due to an eclipse phase. Two further findings suggested caution in making such an assumption; a similar fall occurred in cultures with heated or disintegrated membranes in which the virus did not grow, and there was usually only a slight preliminary fall in titre in cultures of chick-embryo cell suspensions in which the virus multiplied.

The mechanism which accounts for fall in titre of the virus is not known, and conceivably one mechanism could operate in the case of living membranes and another in heated or disintegrated membranes. When virus was incubated at 37° C. in Hanks' solution the loss of infectivity was rapid, about 1 % remaining at 5 hr. Virus in heated membranes, incubated in Hanks' solution, fell to 20–30 % of the original titre in 5 hr. and thereafter dropped much more slowly (Fig. 2), which might be regarded as a physicochemical effect of the tissue-protecting virus to some extent from the still more unfavourable conditions for survival in Hanks' solution itself. Disintegrated membranes might be regarded similarly. This, however, is speculation; the problem requires further investigation. The fact that the fall in titre occurring in living membranes and chick-embryo mince was so closely paralleled by that seen with the non-living tissue, suggested that the processes may be similar, and casts considerable doubt on the idea that the loss of infectivity of virus in the living tissue was part of a life-cycle of the virus concerned with its multiplication.

A different result was obtained with suspensions of chick-embryo cells; the preliminary drop in titre was virtually negligible in some experiments and in most of the others was relatively small, dropping to 65–70 % of the original, a figure which in itself would not support decisively, the idea of an eclipse phase. The experiments, as will be noted, were not entirely uniform with regard to the amount of the preliminary fall in titre, but each experiment was complete in itself and those which showed only a negligible drop in titre have been too many to be disregarded as oddities. The general picture of all experiments with chick-embryo cell suspensions did not favour the interpretation that the drop in titre represented an eclipse phase.

The composition of the suspending medium affected the behaviour of virus in culture particularly when cell suspensions were used. Thus with Hanks' solution the virus grew in chorio-allantoic membrane and minced embryo but not in cell suspension; when chick-embryo extract was added to Hanks' solution it then supported the growth of virus in cell suspensions. This would suggest that something essential for growth of virus was removed during the preparation of cell suspensions and was replaced by 'embryo extract' in the medium. It is not known whether the effect of the medium was exerted directly on the virus or whether it was indirect through an effect on the cells.

Only one strain of virus, adapted to the rabbit skin, has been used for these experiments. It is possible that egg-adapted virus might behave somewhat differently, though it is hardly to be expected that the mechanism of its multiplication would be fundamentally different. The data set out in this paper do not

settle the mechanism of growth of vaccinia virus and further analyses of its growth in cultures of different types are being made.

The studies by microscopy or electron microscopy of intracellular appearances during the growth of vaccinia virus (Bland & Robinow, 1939; Bang, 1950; Wyckoff 1951; Peters & Nasemann, 1953; Gaylord & Melnick, 1953; Morgan, Ellison, Rose & Moore, 1954; Noyes & Watson, 1955; Flewett, 1956) suggests that new virus is formed in certain foci within the cell, and that the units of virus may not at first appear in their final form. But there is no certainty from morphological studies what happens to the virus in the earliest stages after a cell is infected. The tendency has been to assume that it becomes non-infective as an essential part of the process of virus reproduction. This may eventually turn out to be the case, but at present there is no conclusive evidence about it one way or the other. The results reported in this paper suggest that further evidence is required before coming to a final conclusion.

SUMMARY

The growth curve of rabbit skin-adapted vaccinia virus in the chick chorio-allantoic membrane incubated in Hanks' solution showed a drop in titre of virus for about 10 hr. followed by growth. At least 25 % of virus, sometimes more, remained infective. A similar fall in titre was observed in heated membranes in which the virus did not grow and this occurred also when membranes, either normal or heated, were infected and disintegrated before incubation.

The growth curve of virus in minced chick-embryo was similar to that in chorio-allantoic membrane.

Virus in cell suspensions prepared from chick embryo and incubated in a nutrient medium showed only a small loss of infectivity before growth in some experiments and rarely dropped below 65–70 % of the original titre in others.

These results throw considerable doubt on the view that loss of infectivity preceding growth of vaccinia virus should be interpreted as an essential part of a growth cycle.

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