THE PROLONGED COEXISTENCE OF VACCINIA VIRUS IN HIGH TITRE AND LIVING CELLS IN ROLLER TUBE CULTURES OF CHICK EMBRYONIC TISSUES

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PLATE 17

(Received for publication, June 22, 1940)

For the cultivation of viruses *in vitro* three methods (1–4), all of which depend upon the presence of living cells, have been employed: the plasma hanging drop; the flask technique of Carrel which permits the addition of nutritive materials and alterations in gas mixtures; and, finally, the procedure in which fragments of tissue are suspended in a salt mixture to which serum or plasma may be added.

Under the conditions afforded by these methods, the rate of multiplication and decline of a number of viruses have been studied and the effect of these agents upon the cells investigated. It has been demonstrated that during the first few days the amount of virus increases to a maximum which, after a varying period of stability, is followed in nearly all instances by a more or less rapid decrease in viral activity. The time required for the completion of these events varies with the species of virus and with the procedure adopted[1] but, with the exception of tumor-producing agents such as the Rous sarcoma virus and possibly rabies virus,[1] we have failed to find evidence in the literature for survival beyond the 18th day.

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[1] Virus of pseudorabies, 4th to 5th day (5); influenza virus, 5th to 6th day (6); virus of foot and mouth disease, 6th day (7); yellow fever virus, 7th day (8); virus of fowl plague, 8th to 14th day (9); ectromelia virus, definitely decreasing by 12th day (10); vaccinia virus, 8th to 18th day (11–22). In general, it appears that a virus survives longer in the plasma type of medium than in the suspended cell culture. For example, vaccinia virus disappears by about the 9th to 12th day in the latter, whereas in the plasma clot cultures some activity may be retained up to the 18th day (22). Notable exceptions, however, are rabies virus (23) which, although cultivated in the Maitland type of medium and definitely on the decrease the 14th day, was still detectable by the 50th day, and Rous sarcoma virus (24) which remained viable in a culture of fibroblasts for one month.

As the virus increases in tissue cultures it has likewise been shown with many but not all species that injury or death of certain cells intervenes (5, 10, 25–32). Such observations have led to the hypothesis that the disappearance from culture of the active agents may be brought about by their destructive action on these cells (33). But the possibility cannot be disregarded that other causes quite independent of viral action and intrinsic in the techniques employed may lead in certain instances to the more or less rapid cellular death or impairment,[2] and thus indirectly to the decline and disappearance of the virus.

The recent adaptation by Gey of the so called roller tube method to the prolonged maintenance of normal and malignant tissues in an active state appeared to us to offer a means of observing for relatively extended periods the course of events which follows the infection of cells by a virus. It was felt that in this way curves obtained for the rise and fall in concentration of a virus such as vaccinia might form patterns somewhat different from those determined by the standard methods.

The roller tube technique originally was suggested by Carrel in 1913 (36), employed by Löwenstädt in 1925 (37) and subsequently further modified by Carrel (38), Gey (39), Gey and Gey (40) and Lewis (41). In general terms, the basic procedure consists in imbedding tissue fragments, which may be very considerable in number, in plasma distributed evenly over the wall of a test tube. Nutrient fluid is then added and the tube is rotated in a horizontal position at 37°C. Fluids and gas mixtures may be replaced at frequent intervals. It is logical to think that the chief advantage in maintaining the activity of cells for long periods of time obtained by rotation over the "classical" methods lies in the opportunity afforded to the cells more effectively to carry on the respiratory processes since they are alternately exposed to the fluid and gaseous content of the system. Possibly the rotation may also permit a more uniform distribution of harmful metabolic products and nutritive substances.

In spite of its manifest advantages in promoting proliferation and survival of cells the method, to our knowledge, has previously been only once employed for the propagation of virus. While the present investigation was in progress, Gey and Bang (42) reported the cultivation of the virus of lymphopathia venereum in roller tubes.

[2] It has been shown that uninfected embryonic chick tissue incubated for 6 days (28) at 37°C. either in Tyrode's solution alone or in a 1 to 3 mixture of serum and Tyrode's solution or incubated for 12 to 13 days (32) at 37°C. in Drew's liquid loses its capacity to grow when transplanted to hanging drop plasma cultures. Although it is known that fragments of breast muscle from 12 day old chick embryo, when cultured in serum and Tyrode's solution or in plasma and Tyrode's solution, will remain in a state of functional survival for one year (34) without disturbing the tissue other than to add fresh nutrient medium at frequent intervals, it is generally recommended (29, 35) that frequent transplants be carried out with the plasma type of culture when one wishes to obtain continued and prolonged growth and vitality of the tissue.

Materials and Methods

Tissue.—Either minced, whole (eyes removed) 8 to 10 day chick embryos or minced chick hearts from 10 to 12 day embryos were used.

Embryonic Extract.—Minced 10 to 12 day chick embryos (eyes removed) were extracted with an equal volume of Simms' solution (see below) for 20 minutes at 37°C. After centrifugation for 20 minutes at 2400 R.P.M. the supernatant fluid was carefully removed and stored in the ice box. Extracts over 10 days old were not used.

Plasma and Serum.—By cardiac puncture of adult chickens, blood was obtained which served as a source of plasma when 0.02 per cent heparin was added and as a source of serum when defibrinated and centrifuged at 2400 R.P.M. for 20 minutes.

Simms' Solution.—The procedures for the preparation of this solution as given by Sanders (4) were followed, except filtration of mother solution B was effected by means of a Berkefeld N filter. A precipitate which occasionally formed was redissolved by adding carbon dioxide.

Virus.—The strain of vaccinia virus used throughout was obtained from the Massachusetts Antitoxin Laboratory in the form of calf lymph.[3] A suspension of the lymph in hormone broth was prepared and passed through a Berkefeld V filter according to the method of Ward and Tang (43). 0.2 cc. of this filtrate was inoculated onto the chorio-allantoic membrane of the developing chick embryo (Burnet's method (44)). Membranes thus infected were removed after 48 hours. Several passages were carried out in this manner following which the membranes were stored at −20°C. These were employed as a source of virus for inoculating the tissue cultures.

Assembling the Culture.—To a clean 20 × 150 mm. pyrex test tube 5 drops of plasma were added and then spread by means of a Pasteur pipette over the lower two-thirds of the surface of the tube. Fragments of minced tissue were then added by means of a large bore Pasteur pipette slightly curved near the tip. From 30 to 50 pieces of mixed tissue were usually added. Smaller quantities of cardiac muscle (12 to 20 fragments) were employed. As the plasma clotted the bits of tissue were redistributed as desired with the tip of the pipette. After 10 to 15 minutes any excess plasma which collected in the butt was removed. 1.6 cc. of nutrient fluid consisting of Simms' solution 7 parts, embryonic extract 2 parts, chicken serum 1 part were then added, and the tube gently rolled with a rocking motion which insured thorough wetting of the tissue-plasma surface. The tube was then sealed with a one-holed rubber stopper fitted with a short piece of pyrex tubing which in turn was closed with a rubber vaccine cap. Quantities of embryonic extract less than that noted were not found to be as satisfactory in stimulating growth.

Incubation.—Cultures were placed horizontally in a rotating device which turned 8 to 10 times every hour. This apparatus consisted of a large wooden cylinder into which a number of holes were drilled to receive the culture tubes. The cylinder, mounted on a shaft, was rotated constantly at the desired speed by means of an electric motor connected through reduction gears. The apparatus was kept in the incubator room at 37°C.

Routine Care of Cultures.—At approximately the same time each day the nutrient fluid was removed as completely as possible through the small pyrex tube by means of a Pasteur pipette and 20 cc. of air which had been drawn through sterile cotton with a

[3] For the history of the strain of vaccinia see Robinson (45).

30 cc. syringe were then introduced. 1.6 cc. of nutrient fluid were added and the tube was again sealed and returned to the rotator without delay. To obtain optimum conditions for growth, it was necessary to change the fluid each day. The metabolic processes of the rather large amounts of tissue employed usually decreased the pH from an initial value of about 7.8 to 7.0 or below after 24 hours. Continued exposure to this acid medium was found to retard growth and induce degenerative changes.

Approximately every 5 days the plasma coagulum required "patching." With the tube drained of its fluid contents, 4 drops of plasma were added and quickly distributed by rolling the tube with a rocking motion. If the plasma did not clot readily, as often occurred when the culture was over 20 days old, 2 drops of embryonic extract were added.

Addition of Virus.—The infected chorio-allantoic membranes (see above) were triturated with a few drops of infusion broth and then suitably diluted with Simms' solution. Nutrient fluid was then prepared in which the required volume of this suspension replaced an equivalent portion of the Simms solution. The amount of virus present was later determined by titration (see below). 1.6 cc. of the inoculated nutrient fluid were added to each tissue culture in place of the usual medium and removed after 24 hours. In no instance was a further addition of virus made.

Titration of the Virus.—Quantitative estimations of the amount of virus present in samples of the nutrient fluid removed daily and in the tissues at the termination of the culture were obtained in the following manner. Materials to be titrated were stored at −20°C. at which temperature no loss of viral activity was observed for at least 20 days. Tissue with the plasma coagulum was scraped off the surface of the tube and ground in a mortar with the addition of 1.6 cc. of infusion broth. This suspension was then stored at −20°C. A series of tenfold dilutions of these various materials were made in infusion broth and 0.1 cc. of each dilution injected intradermally into the shaven skin over the back and upper portions of the flanks of a normal rabbit. A clean pipette was used for each dilution. The highest dilution producing a papular erythematous lesion on the 5th day was regarded as the end point. Injections of fluid and tissue suspensions derived from cultures to which no virus had been added regularly failed to give any recognizable reactions.

Further Comment on Cultural Procedures.—As a result of the patching, no definite changes were observed either in the growth of the tissue or the quantity of virus in the fluid or tissue.

The cultures were examined daily under the low power objective and the condition of the cells was noted.

In certain instances a record of the growth of the tissue was obtained by the following method. A strip of celluloid film was attached to the tube by means of rubber bands. The tube was then placed over the light in a box used for reading agglutination tests. With the aid of a hand lens held in a clamp, the outline of the tissue fragment lying beneath the film was then scratched on the latter with a sharp dissecting needle. Successive tracings of the same fragment could in this way be recorded on the same strip.

Tissue for the preparation of stained total mounts was obtained by a slight modification of the method described by Gey (39, 40). One or two cover slips (12 × 50 mm.) were placed in a clean culture tube and sealed to the wall by running beneath them melted 3.0 per cent agar in saline. The tube, fitted with a cotton stopper, was then incubated at 37°C. for 24 hours to drive off the water vapor which at first condensed on the cover slip and interfered with the adhesion of the plasma if the culture was assembled

at once. In preparing the culture the routine procedure was followed except that tissue fragments were placed on the surface of the cover slips. At any time during the course of an experiment a cover slip could be withdrawn, stained and examined.

With care contamination became very infrequent considering the very large number of manipulations to which a culture was subjected over a period of many weeks.

Morphological Technique.—In addition to the daily observations of the living tissue, studies were made of fixed and stained total mounts, materials for which were obtained in either of two ways: (*a*) By use of the cover slip technique described above. On removal, the cover slip was placed in fixing solution and then cut into three pieces for staining by different techniques. (*b*) By removal of intact sheets of the plasma coagulum from the wall of the tube. After draining off the nutrient fluid, fixing *in situ* and dehydrating to 70 per cent alcohol, it was possible to wash or scrape off pieces of the plasma coagulum containing the proliferating tissue. This manipulation was most successful on cultures over 14 days old, as younger cultures adhered firmly to the tube.

Zenker's fixative was used throughout. After many trials of a variety of stains, four were selected for routine use: Alum-hematoxylin and eosin, Downie's modification of Mann's stain (46), Goodpasture's carbol-fuchsin methylene blue stain (47) and a modification of the acid-fuchsin stain described by Buddingh (48) in which Loeffler's methylene blue was used as the counter-stain. Of these, the last proved most satisfactory for the staining of inclusion bodies.

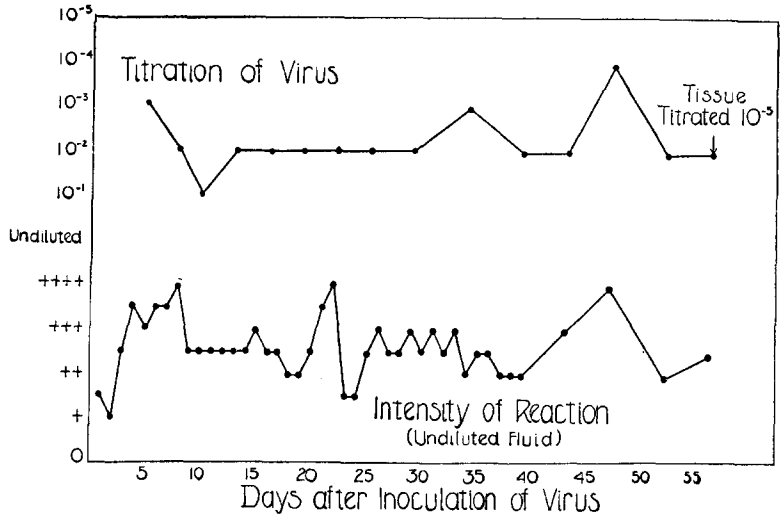
I. Quantitative Determinations of the Virus in the Fluid and Tissue at Various Stages of Cultivation

To investigate the possibilities of the roller tube method for the propagation of vaccinia virus, it was obviously necessary to obtain, at varying intervals, quantitative estimations of the viral content of the fluids and of the tissue. The following experiments were designed to secure this essential information.

(*a*) *Fluid.*—The quantity of virus present in many of the nutrient fluids removed each day was determined either by titration or by injection of the undiluted material. Twenty-seven cultures maintained for varying periods[4] and comprised either of mixed tissue or of heart muscle were studied in this manner. In Text-fig. 1 are summarized the data obtained by titrating samples of the fluid from a culture of mixed tissue inoculated on the 5th day of cultivation with a suspension of virus exhibiting a potency of about 10^{-4}. It will be noted that considerable amounts of virus were

[4] One for 59 days; 1 for 56 days; 4 for 40 days; 1 for 37 days; 1 for 30 days; 1 for 25 days; 3 for 24 days; 1 for 20 days; 4 for 16 days; 1 for 15 days; and 9 for less than 15 days. The infecting dose of virus has varied from 10^{-1} to 10^{-6}. The tissue has been infected as late as the 9th day of cultivation and, on one occasion, the tissue was soaked in the virus suspension before implantation in the culture and yet the subsequent course of events, so far as the amounts of virus in the fluids and tissues were concerned, was not materially altered

present throughout a period of 56 days. Its titre varied between 10^{-1} and 10^{-4}, but in most instances was 10^{-2}. Of importance in evaluating these results was the fact that the highest titre was obtained toward the close of the experiment, *i.e.* on the 46th day, since this raises the question as to whether continued production of the virus took place during this prolonged time. The answer to this query is of course of great interest and will receive further attention subsequently in this paper. It should be pointed out that the amount of virus in the tissue was greater on the 56th



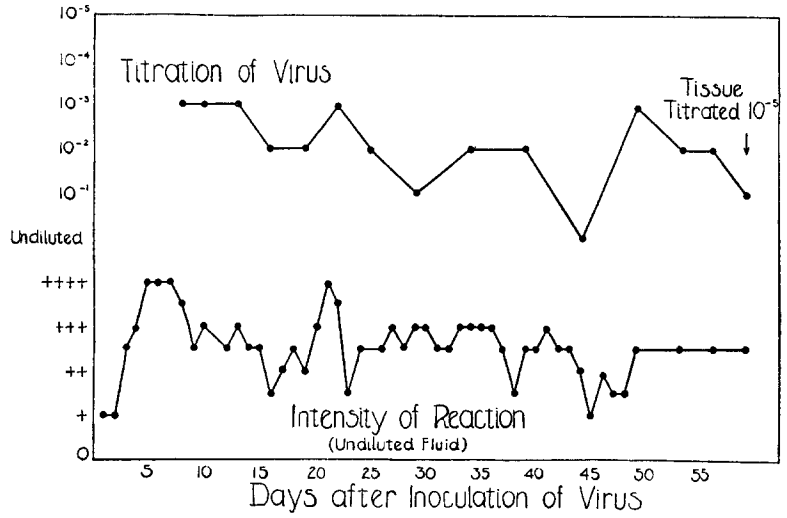
TEXT-FIG. 1. Quantitative estimations of vaccinia virus in the nutrient fluids from cultures of 9 day chick embryonic tissue.

Upper curve based on data obtained by titration of the fluids. Lower curve indicates the intensity of the reaction in the rabbit's skin resulting from the injection of undiluted nutrient fluids.

day than that which was introduced as the inoculum in spite of the considerable amounts removed in the fluids. It should be mentioned that this culture became contaminated with diphtheroid bacilli on the last day of the experiment. There is no reason to believe, however, that this affected the results of the titration.

In Text-fig. 2 is given the record of a similar experiment but one in which the culture consisted only of cells developing from fragments of the hearts of 11 day chick embryos infected on the 9th day of cultivation. The inoculum was the same as that of the previous experiment. This choice of tissue was made not only with the object of simplifying the conditions somewhat by reducing the number of cell types present, but also to obtain more

satisfactory preparations for showing the effect of the virus on fibroblasts (see section on morphology). Close similarity to the curve of Text-fig. 1 is apparent. Virus was demonstrated in each of the fluids, and the tissue on the 59th day, when diluted 10^{-5}, gave a typical reaction in the rabbit's skin. A calculation of the dilution of the inoculum demonstrates the impossibility that the virus repeatedly found in the fluids could have consisted of remnants of the virus originally introduced. During the period of nearly 9 weeks several of the original pieces of cardiac muscle main-



TEXT-FIG. 2. Quantitative estimations of vaccinia virus in the nutrient fluids from cultures of cardiac muscle from 11 day chick embryos.

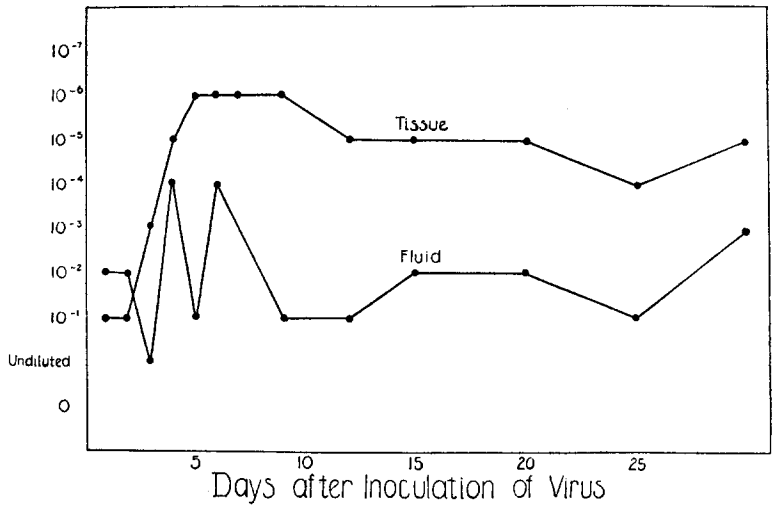
Upper curve is based on data obtained by titration of the fluids. Lower curve indicates the intensity of the reaction in the rabbit's skin resulting from the injection of undiluted nutrient fluids.

tained rhythmical contractions,—an observation which not only indicates the efficiency of the method, but also clearly shows that cardiac muscle cells are not seriously impaired by continued exposure to the virus of vaccinia. Since chick heart is composed exclusively of cells of mesodermal and entodermal origin (49), we have further evidence in support of those authors who, largely on the basis of morphological observations, have asserted that vaccinia virus may invade and multiply in cells other than the epithelial elements (50, 51). Moreover, it is generally agreed that the growth of new cells from chick heart muscle consists only of fibroblasts (29). Accordingly, in these findings is the implication that this type of cell may support growth of the virus, a conception that has been denied by cer-

tain workers (12, 29, 52). Further evidence for the susceptibility of the fibroblast will be found in the section on morphology.

(*b*) *Tissue.*—In the foregoing experiments it was found that the virus was present in the tissue as distinguished from the fluid after prolonged cultivation. It therefore seemed desirable to obtain more complete data concerning the quantity of virus in the tissue at various intervals following inoculation.

Thirteen cultures containing approximately the same quantities of chick heart from 12 day embryos were assembled and inoculated at once



TEXT-FIG. 3. Quantitative estimations of vaccinia virus in the tissue and fluid of individual cultures selected at various intervals from a series of similar cultures of cardiac muscle obtained from 11 day chick embryos. Ordinates indicate highest dilution of the materials giving a definite reaction in the rabbit's skin.

with a virus suspension which titrated 10^{-2}. All were maintained in the routine manner with daily changes of nutrient fluid. At the intervals noted in Text-fig. 3 the potency of the virus in the tissue and fluid from one of the cultures was determined. In spite of unavoidable individual differences which may have existed, the curves based on the data thus obtained probably represent fairly accurately the amounts of virus which might be present in each component of a culture at a given time during the period of cultivation.

It is apparent that the virus rapidly multiplied in the tissue to reach a maximum by the 5th day, which appears to have been maintained for at least 4 days when a tenfold decrease occurred. This may represent a

real diminution due to altered conditions in the culture, although it could be attributed to experimental errors such as differences in the reactivity of the skin of the different rabbits used in the titrations, or discrepancies in the number of living cells in the culture.

In 12 additional experiments in which whole embryonic chick tissue was used, we have regularly obtained titres varying from 10^{-5} to 10^{-7} with a majority at 10^{-6}. The highest titre was reached in the case of a culture incubated for 24 days. Whatever factors may eventually be found to account for the apparent maximum in Text-fig. 3, it is evident that a level is soon attained which is sustained. From the records in Text-figs. 1 and 2 one may perceive that this level persists for as long as 59 days, and we have no reason to doubt that it might be further prolonged.

When the curve of fluid titrations is compared with that of the tissues a rough parallelism is noted, but it is clear that a constant ratio between the quantities of virus in the two components did not exist. In accordance with the experience of others (5, 7, 10, 19, 27, 53), we have almost constantly found more virus in the tissue than in the fluid.

The finding that virus persisted for weeks in large amounts in our cultures was, of course, in disagreement with the observations of many workers who have found that with the usual methods of tissue culture, the virus rapidly reaches its maximum and soon thereafter begins to decline and is nearly always gone by the 18th day of cultivation.[1] Therefore, we next turned our attention to the possibility that our results might not depend on the circumstance that the method of tissue culture which we employed offered the tissue a better opportunity to survive and remain in fairly good condition for long periods, but on some fortuitous circumstances inherent in the technique. These control experiments will now be presented.

II. Control Experiments

(*a*) *The Possible Effect of the Addition of New Cells to the Culture via the Nutrient Fluid or Plasma.*—The routine manner of preparing the embryonic extract, serum and plasma did not result in entirely cell-free fluids. This fact led to the thought that the occasional addition of new cells might have furnished a means by which the virus was preserved or increased.

To investigate this possibility, a culture of 9 day chick embryonic tissue was exposed to virus (potent in a dilution of 10^{-2}) for 24 hours, and was handled in the routine manner except for the following modifications which were introduced to avoid the addition of cells. The serum and plasma prepared in the usual way, were centrifuged for 20 minutes at 2400 R.P.M. and the supernatant fluid cautiously removed. The centrifugation was

then repeated. The standard embryonic extract was centrifuged at 3500 R.P.M. for 30 minutes. The supernatant fluid, carefully removed from the small amount of sediment, was twice frozen at −20°C. and thawed at room temperature. It was again centrifuged at 3500 R.P.M. for 30 minutes and then used for the composition of the nutrient fluid. The small amount of sediment from the last centrifugation examined microscopically contained no cellular elements but consisted of an amorphous debris.

The culture supplied with plasma and nutrient fluid made up with these materials which we considered to have been free of cells, was maintained for 24 days. Virus was demonstrated daily in the fluid and at the end of this time the virus in the tissue was active in a dilution of 10^{-7}. For comparison another culture assembled and infected in the same manner but nourished with fluid prepared in the routine way, also yielded virus each day and at the termination of the experiment, tissue which was active in a dilution of 10^{-6}. No difference in the state or activity of the proliferating cells was noted in the two cultures.

The conclusion, based on the foregoing results, that the continued presence of virus was not dependent on the addition of viable cells from time to time, was given further support by the following experiment which likewise demonstrated the rapid disappearance of virus from a system otherwise identical with that employed as routine but which did not contain tissue:—

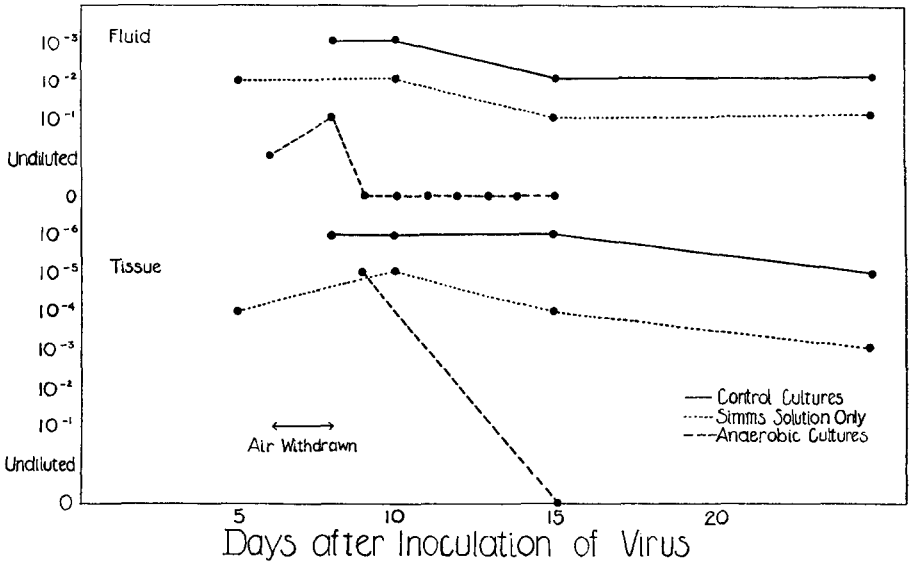
The usual amount of plasma was spread on the walls of two tubes and clotting induced by the addition of a few drops of embryonic extract. A suspension of virus (titre: 10^{-2}) in nutrient fluid prepared in the ordinary way was introduced. Fresh fluid was added each day for 20 days. At no time were cells observed in the tube nor did any change in the hydrogen ion concentration occur. Virus could not be demonstrated in the fluids after the 3rd day.

(*b*) *The Duration of Viral Activity Following the Death of the Cells.*— The persistence of virus in the tissue for many weeks, associated with a constant dissemination of small quantities into the fluid, might be thought to depend, after an early increase to a maximum, merely upon its survival independent of any essential conditions provided by living cells. This possibility received some experimental support from the observations of Amies (54) who stated that vaccinia virus suspended in broth and kept at 37°C. was still demonstrable after 38 days, although in greatly reduced amounts.

The following experiment was carried out to determine whether the virus could survive in cultures such as we have employed after the cells had been killed by oxygen lack:—

Six cultures containing approximately the same amount of minced 5 day chick embryo were inoculated with a suspension of virus exhibiting a titre of 10^{-1} and were subsequently treated in the routine manner. In four of them at various periods the virus

content of both tissue and fluid was determined. These data are summarized in Text-fig. 4 which shows that the curve of viral increase and persistence followed the usual course. On the 6th day of cultivation the air in the two remaining cultures was largely removed by suction, the tubes sealed and left in the rotator for 48 hours. Then air was admitted and nutrient fluids changed. On the following day (9th day of cultivation) no alteration in the pH of the fluids could be recognized, and microscopic examination revealed degenerative changes in the cells and no indication of cellular activity or growth. One of the cultures was then sacrificed. In the fluid virus could not be demonstrated, but the end point of the diluted suspension of tissue was 10^{-5}. Thus anaerobic condi-



TEXT-FIG. 4. The effect of reduced activity of the tissue and the death of the tissue through oxygen lack on the quantity of virus present in tissue and fluid. Ordinates indicate highest dilution of the materials giving a reaction in the rabbit's skin. For explanation, see text, Experiments II *b* and II *c*.

tions for the 48 hour period had not markedly reduced the quantity of virus but had apparently brought about the death of the tissue. The remaining "anaerobic" culture was maintained for 6 days longer in the routine manner when the tissue and fluid were removed and titrated. As reference to Text-fig. 4 will show, in neither could virus be revealed nor, indeed, had it been present in the fluids during these 6 days. Furthermore, throughout this interval there was no indication of tissue viability since the cells began to disintegrate and the pH of the fluids remained constant.

Since virus had been demonstrated in the fluids of this particular culture, both on the day air was withdrawn and the day when it was readmitted, it seemed certain that this culture had contained virus and that the virus had survived the withdrawal of air which had led to the death

of the tissue. This conclusion is also supported by the fact that the culture which had been sacrificed contained virus in the tissue potent 10^{-5}. Accordingly, we may conclude that the virus present in the culture became inactive in less than 7 days following the death of the cells. The continued presence of virus in high titre in our cultures, therefore, depends upon the coexistence of living cells.

(*c*) *The Relationship between the Activity of the Cells and the Quantity of Virus Produced.*—Although the preceding experiment demonstrated that virus would not persist in our cultures unless the cells were alive, the question still remained whether, for the maintenance of the virus at a high level, cell viability alone was essential, or whether, in addition, a certain degree of cellular activity was required.

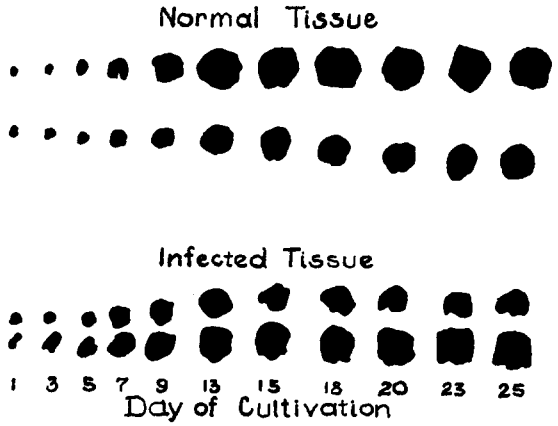
To investigate this possibility, eight cultures of minced 9 day chick embryo were infected with virus active in a dilution of 10^{-1}. Four of them were nourished with the usual fluid (these were the same cultures which served as controls in the experiment described in section II *b*). Simms' solution only was added after the first 24 hours to the remaining four which, however, in all other respects were treated in the same manner. At varying intervals the virus content of both tissue and fluid from a culture in each group was determined. The results of these titrations are depicted in Text-fig. 4. It will be noted that in the cultures to which Simms' solution alone was added, the virus increased more slowly, never attained the concentration exhibited by those maintained according to the routine procedure and decreased to a relatively low titre by the 25th day. Growth of new cells was approximately the same in all the cultures during the first 8 to 10 days in so far as extension in one plane was concerned, but it was evident that the new cells were only one layer thick in the tube receiving Simms' fluid, whereas in the controls multilayered tissue developed after a short time. By the 14th day the growth was definitely less in the remaining tubes which had received Simms' solution and in the last culture of the group it was scanty compared with that in the tube which had been nourished with embryonic extract and serum. In contrast with the controls, the daily decrease in pH in these cultures, although readily apparent, diminished as cultivation was continued. This was interpreted as an index of reduced metabolic activity, and also, possibly, of a diminution in the number of viable cells. It should be emphasized, however, that, although diminished, the change in pH which was taking place even at the end of the experiment, as well as the appearance of the cells, indicated that many of them remained alive.

Thus, it appeared that when the activity of the cells was reduced by unfavorable environmental factors, which, however, did not lead to the death of all of them, the quantity of virus in the culture decreased significantly. In this connection the findings of Plotz (55) are pertinent. In the case of the viruses of fowl plague and vaccinia he obtained larger yields when proliferating cells were present than when living but non-proliferating cells formed the basis of the cultures.

III. Effect of Virus on the Cells

The unexpected persistence in roller tube cultures for nearly 2 months of virus in high concentration revealed by the foregoing experiments, led us naturally to attempt to gain some insight into the processes which were taking place during this sustained association between virus and the cells. Accordingly we proceeded to investigate the influence of virus upon the growth of the tissue, and to study the morphological changes which it induced at various stages of cultivation.

(*a*) *Comparison of the Growth of Tissue in Infected and Non-Infected Cultures.*—The lateral extension of the cells growing out from certain of the



TEXT-FIG. 5. The failure of vaccinia virus obviously to influence the growth of cardiac muscle tissue from 9 day chick embryo. Two upper rows show the lateral extension of two fragments of tissue to which no virus was added. The lower rows are tracings of the margin of the visible extension of new cells in a culture which contained virus throughout the period of observation.

tissue fragments in the cultures of chick heart muscle employed in Experiment I *b* were measured at intervals of 2 to 4 days by means of the celluloid film technique previously described. The records thus obtained for two fragments grown in a culture inoculated with virus titrating 10^{-2} are presented as examples in Text-fig. 5. The tracings of the growth of two pieces of heart muscle from a culture to which no virus was added are included for the purpose of comparison. It is evident that no significant differences existed either in the rate or extent of the growth in the infected as contrasted with the normal tissues, although reference to Text-fig. 3 will show that increase and persistence of virus in large amounts occurred. It should be pointed out that while Text-fig. 5 gives the im-

pression that lateral extension continued actively for only 12 to 14 days in either culture, microscopic observations made daily upon the tissues *in situ* and subsequently in stained preparations, revealed a continued diffuse spread of cells (see section on morphology) which, apparently, was not sufficiently defined to lend itself to measurements in the gross.

It is important, with respect to this experiment, to remark that a modification of the growth pattern may be introduced if a very large infecting dose of virus is employed. Thus, in one case in which a suspension titrating 10^{-6} was used, widespread destruction of tissue ensued within 24 hours. Certain cells remained uninjured, however, and by proliferation eventually more than replaced the tissue which was lost. Moreover, subsequently the virus in the fluids and tissue was demonstrated in quantities equivalent to those obtained in cultures inoculated with smaller doses.[5]

(*b*) *Reproduction of Cells in Explants from an Infected Culture.*—That certain cells not only may survive for an extended period in an environment heavily infected with virus but that they may also still retain the capacity to reproduce under these circumstances is shown by the following experiments:—

A culture of minced 9 day chick embryo was inoculated on the 6th day of cultivation. On each of the following 37 days virus was found in the fluid. At the end of this time the virus in the tissue was potent in a dilution of 10^{-5} and 16 small pieces of tissue were removed. These were employed to inaugurate a new culture to which no fresh tissue or virus was added. These explants were carried along in the routine manner for 32 days. Many of the fluids removed daily were titrated. As is manifest in Text-fig. 6, virus was demonstrated in decreasing amounts for the first 3 days. During the 11 following days the fluids were found to be inactive. But on the 16th day of cultivation, virus reappeared and was thereafter present in the majority of fluids tested. In most of the explants no signs of activity were noted, but in three or four of them a very slow growth of granular cells took place for the first 14 days when a moderate increase in proliferation appeared. Growth about these fragments continued progressively albeit slowly until the termination of the experiment at which time a definite sheet of new cells was plainly evident in the gross. An emulsion of the tissue with the exception of one fragment reserved for staining, was found to be active in a dilution of 10^{-3}. This relatively low titre may well have been due to the very small amount of living tissue available.

We may conclude from this experiment that cells removed after several weeks from an infected culture retain the capacity to proliferate. It likewise strongly suggests that this continued cell division is accompanied by production of virus.

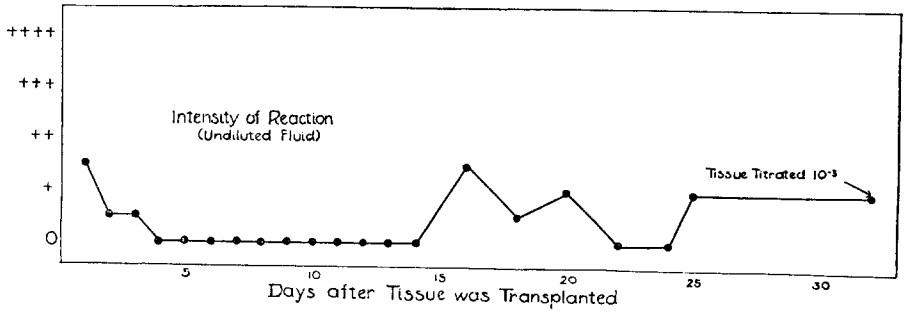
[5] For further observations on the effect of large infecting doses of virus, see section on morphology.

(*c*) *Morphology.*—The results of the foregoing experiments are supplemented and confirmed by morphological observations on living and stained tissues at various stages of cultivation.

1. Observations on the Living Tissues.—

In general, the observations on the living tissue at various periods during cultivation may be summarized as follows:—

In both the virus-infected and uninfected minced whole chick and chick heart cultures, a rapid, orderly, radial type of growth in which fibroblasts predominated, took place during the first 10 to 14 days. Thereafter, the rate of growth gradually decreased and the orderly arrangement was superseded by an aimless spread of individual cells throughout the plasma clot. At the end of a period of 30 to 40 days, most of the plasma



TEXT-FIG. 6. The presence of vaccinia virus in the nutrient fluids removed from tissues explanted from a culture of 9 day chick embryonic tissue inoculated with virus 37 days previously. Ordinates denote the intensity of the reaction in the rabbit's skin following the injection of the undiluted fluid (see text, Experiment III *b*).

clot had been invaded by growing cells. This describes the morphological picture noted in all our cultures which received inocula varying in titre from 10^{-1} and 10^{-4}. In two cultures, however, to which a suspension of virus titrating 10^{-6} was added on the 6th day of cultivation, indication of widespread injury to the cells was visible after 24 hours, characterized by necrosis of many elements and a rounding up of others which soon became extremely granular. Some of these round cells, however, as judged from protoplasmic emanations which soon appeared, remained alive and ultimately, from groups of them, fibroblast-like cells were seen to emerge. Almost at once, however, new growth of fibroblast-like cells was noted in certain areas which persisted at a somewhat reduced rate for 20 days and later became more active, resulting in a fairly extensive laying down of new tissue. This isolated observation suggests that the initial effect of the virus on the cells may vary with the concentration employed, but the possibility must be considered that factors entirely unrelated to the action of the virus may have intervened. A final decision must await further experimentation.

In all cultures, from the 3rd to 8th day of growth, numerous highly refractile cytoplasmic granules or vacuoles appeared; these closely resembled the fat granules described and pictured by Simms and Stillman (58). Although in one experiment such granules

appeared earlier and in larger numbers in the virus-infected tissue than in the control tissue, no definite relationship could be discerned between the presence of the virus and the granules. A rough correlation was found between the age of the culture and the size of the vacuoles. In young cultures, the actively growing cells contained many small granules, while in cultures over 40 days old, it was common to see a vacuole occupying most of the cytoplasmic area, which so displaced the nucleus of the fibroblast that the latter resembled a fat cell. Such cells showed no signs of active growth. Although granulization (or vacuolization) was a common feature, all cultures invariably showed at all stages a number of normal appearing non-granulated fibroblasts.

2. Observations on Stained Preparations.—

In stained preparations, proliferating cells, chiefly of the fibroblastic type, were noted. These were present in all the infected cultures, even as late as the 59th day of cultivation. Mitotic figures, however, were more abundant in 5 to 10 day cultures than in those cultivated for a longer period. In those over 50 days old, mitotic figures, although present, were rare.

Cytoplasmic inclusion bodies were characteristic of material from vaccinia-infected cultures, while no inclusions were observed in uninoculated tissue. These bodies were found in preparations as early as 2 days after inoculation, were extremely numerous from the 5th to the 10th day and were seen in cultures 59 days old. The inclusions occurred anywhere in the cytoplasm, but frequently were paranuclear in position. They presented two morphological extremes with intergrading forms. At one extreme, the bodies consisted of an acidophilic clump of minute granules which stained heavily with acid-fuchsin and poorly with the samples of eosin tried. Such bodies were irregular in shape and size, varying from a clump consisting of a few granules, to one which occupied nearly the whole cytoplasmic area of the cell. At the other extreme, the inclusions consisted of dense, semi-homogeneous, irregularly oval structures which showed less variation in size and were less acidophilic than the granular masses. They stained pink with acid-fuchsin and pale lavender with hematoxylin and eosin. Frequently, more than one body was present in a single cell. The cytoplasm immediately adjacent to the inclusion was often stained faintly so that some of the bodies appeared to rest in poorly defined vacuoles. Inclusion bodies were never seen in cells showing mitotic figures.

Inclusions were most common in the fibroblast-like cells and also occurred in rounded monocytic cells, but it is impossible to state definitely that these represent two distinct cell types. No morphological evidence was obtained regarding the effect of the virus on epithelial cells. The irregular distribution of inclusion bodies in any particular specimen was a striking and constant feature. Thus, in one area numerous inclusions might be found, whereas in another a few millimeters away composed of similar cells, none might be observed. Inclusions were usually seen in areas of actively growing fibroblastic cells and were almost never found in the slowly growing cells which contained extremely large vacuoles (fat-like cells). In some areas, however, in which a large number of the cells contained inclusion bodies, some of the fibroblastic cells appeared to be undergoing fragmentation and others had fused to form large multinucleated cells. It is impossible to say at present whether or not these changes were the result of the action of the virus on the host cell. (For photographs showing inclusion bodies and the condition of the cells after 2 to 3 weeks cultivation, see Figs. 1 to 4.)

The data described in this section show that when moderate doses of virus are employed as inocula, the proliferation of new cells is not manifestly affected throughout a period of many weeks although the virus soon reaches a high concentration. In spite of this fact many cells are presumably attacked by the virus as indicated by the presence of intracytoplasmic inclusion bodies. Since these occur chiefly in cells resembling fibroblasts and since the latter represent the principal cell type which grows in cultures of fragments of chick hearts, it is strongly suggested that the fibroblast-like cell is capable of supporting the growth of the virus. We cannot state, however, that infection of these cells by the virus leads to their destruction. It is possible that they may survive its attack and even continue to proliferate.

Some of the short-term observations of other authors are suggestive of the conditions encountered in our cultures. Beard and Rous (56), working with cultures of Kupffer cells infected with vaccinia virus, noted that the virus increased rapidly and survived for at least 7 days without exerting any obviously injurious effect on the cells, which remained in excellent condition and continued to take up neutral red even at the termination of the experiment. Rivers, Haagen and Muckenfuss (57) showed that fragments of chicken kidney suspended in Tyrode's solution and infected with the same virus retained, at the end of 5 days, the capacity to proliferate when transferred to plasma clots. Our own findings demonstrate that certain cells remain viable for 59 days at least when constantly exposed to this virus.

DISCUSSION

The most significant fact, we believe, which emerges from this study is that, under the particularly favorable circumstances afforded by the roller tube method for the prolonged cultivation of tissue, the quantity of virus after an early increase to a high concentration remains in large amounts for as long as 9 weeks. Nor does this in all probability represent a limit. Moreover, in spite of this continued presence of the active agent, many of the cells remain alive and undergo division. As we have previously remarked, these observations are not in accord with the majority recorded by others concerning the persistence of various viruses in cultures carried out by different techniques (5, 10, 25–32).[1] But a minority of reports have afforded an indication that survival of a virus *in vitro* may not always be relatively brief nor its action on cells necessarily destructive. We have already referred to the work of Beard and Rous (56) and Rivers and his coworkers (57) who showed an association of vaccinia virus and living

cells for 5 to 7 days and to Carrel's (24) demonstration that the Rous sarcoma virus persisted for 1 month in tissue culture without obvious injury to the cells. In addition Minerwin and Schmerling in 1926 (16), Haagen in 1928 (59) and Hecke in 1930 (7) found that the time of survival of a virus in a tissue culture was longer when the infected tissue was transplanted to a fresh medium than when the original culture was allowed to run its course. In this way, Minerwin and Schmerling were able to demonstrate vaccinia virus in their explants at the end of 27 days.

The precise nature of the process which is responsible for the prolonged coexistence of virus and proliferating tissue, in our experiments, still remains undefined. We may, however, formulate two hypotheses which could account for it and then proceed to inquire which appears to be most in accord with the experimental facts. On the one hand the virus, after an early increase in, and destruction of the susceptible cells present at the time of inoculation, may be preserved in an extracellular environment without further multiplication under the special conditions afforded by other cells which, completely insusceptible to the action of the virus, remain alive. Certain cells might, however, be partially susceptible and continue to harbor virus without themselves undergoing destruction. On the other hand, multiplication of the virus may be indefinitely sustained through the proliferation of cells which, when infected, may survive. Or virus, after destroying the cells which have permitted it to increase, may enter newly developed completely susceptible cells which, in some manner, have escaped infection. If continued multiplication of virus were taking place, to explain the constancy of titre which has been experimentally determined, the assumption would have to be made that a fixed proportion of the virus released from the infected cells became inactive.

No positive evidence in favor of the first alternative, *i.e.* simple preservation, is to be found in our experiments. Against it is the demonstration that when the metabolic activity and proliferation of the tissue is diminished, although living cells be still present, the titre of the virus decreases. Furthermore, experiments (61–64) reported in the literature in which the survival of various species of virus was studied when these agents were separated by semipermeable membranes from non-infected living tissues, although showing that under these conditions, the virus may persist somewhat longer than when living tissue was absent, indicate that this preservative effect of living non-infected cells was not sustained.

In favor of the hypothesis of continued multiplication is the fact that a constantly high level of virus is only maintained when conditions for cell

metabolism and multiplication are continuously afforded. Moreover, cells explanted from a culture which had been infected for 5 weeks retained the capacity for proliferation which was accompanied by the appearance of virus in the culture,—an observation which not only strongly supports the conception of constant production of virus, but likewise indicates that the cells responsible for its production are not immediately destroyed. Finally, the presence of inclusion bodies in cultures of 9 weeks duration carries with it, we believe, the inference that virus is still being produced.

If the concept of continued multiplication be correct, there is much ground for believing that the production of virus is mediated by the fibroblasts. Thus, as nearly as one can determine from the examination of both living and fixed preparations of mixed embryonic chick tissue, it is these cells which come to predominate and in which mitotic figures are observed. Furthermore, when fragments of cardiac muscle are employed from which fibroblastic cells only appear to grow out, increase and production of virus is in every way comparable to that which occurs in cultures composed of minced whole embryos. In addition to these observations, characteristic cytoplasmic inclusion bodies have been seen throughout the period of cultivation in cells having all the morphological attributes of fibroblasts. Even though the virus should soon cease to multiply and merely thenceforth survive, these data indicate that the fibroblast-like cell in all probability supplies an essential factor in the conditions which make survival possible.

In addition to raising these questions in respect to the virus-cell relationship as it exists *in vitro* when cultivation is prolonged, our experiments have shown that the roller tube method provides a relatively simple means of obtaining excellent yields of the virus of vaccinia which, of course, is among those viruses more readily cultivated. Gey and Bang (42), however, have attained similar results with the more delicate virus of lymphopathia venereum. We confidently expect that as other viruses are tested, the technique will be found to offer certain advantages over those methods of tissue culture in common use for the propagation of certain viruses.

Since the roller tube method appears to afford conditions for extended observations, it may well be adapted to the investigation of problems in resistance and immunity to viral agents; to detailed study of the effect on cells after long contact with the virus; and possibly to the revelation of "masked" virus in tissue obtained from convalescent animals or in those where it has heretofore been impossible to demonstrate the active agent, as in the case of the rabbit cancers originating from papillomas due to the

action of Shope's virus. With the introduction of modifications tending toward simplification of the technique, it might likewise offer a practical method for the preparation of vaccines.

In conclusion, we wish to make it clear that the experimental results reported in this communication were obtained with one species and one strain of virus and under one set of experimental conditions. To apply indiscriminately these findings to viruses in general would manifestly be unwarranted.

CONCLUSIONS

1. The virus of vaccinia in so called roller tube cultures of mixed embryonic chick tissue rapidly increases to maximal titre.

2. Under these conditions the quantity of virus in the tissue remains at or near the maximum for at least 9 weeks and considerable amounts are present in the fluids removed each day.

3. The same results are obtained when only fragments of embryonic chick heart are employed.

4. Many, though not necessarily all, of the cells in infected cultures remain alive and retain the capacity to proliferate. The presence of these living cells is essential for the persistence of the virus.

5. No apparent differences in the rate or amount of growth of cells in infected as contrasted with non-infected cultures can be discerned in the gross.

6. It is suggested, but not proved, that virus is continually being produced rather than simply preserved throughout the period.

BIBLIOGRAPHY

1. Hallauer, C., Die Züchtung der Virusarten ausserhalb ihrer Wirte. Die Viruszüchtung im Gewebsexplantat, in Doerr, R., and Hallauer, C., Handbuch der Virusforschung, Vienna, Julius Springer, 1938, pt. 1.
2. Haagen, E., Züchtung der Vira, in Gildemeister, E., Haagen, E., and Waldmann, O., Handbuch der Viruskrankheiten, Jena, Gustav Fischer, 1939, **1**.
3. Plotz, H., Culture des virus, in Levaditi, C., and Lépine, P., Les ultravirus des maladies humaines, Paris, Librairie Maloine, 1938, **2**.
4. Sanders, M., *Arch. Path.*, 1939, **28**, 541.
5. Traub, E., *J. Exp. Med.*, 1933, **58**, 663.
6. Magill, T. P., and Francis, T., Jr., *J. Exp. Med.*, 1936, **63**, 803.
7. Hecke, F., *Zentr. Bakt., 1. Abt., Orig.*, 1930, **116**, 386.
8. Haagen, E., *Zentr. Bakt., 1. Abt., Orig.*, 1933, **128**, 13.
9. Plotz, H., *Compt. rend. Acad. sc.*, 1933, **196**, 1545. Hallauer, C., *Z. Hyg. u. Infektionskrankh.*, 1931, **113**, 61.
10. Downie, A. W., and McGaughey, C. A., *J. Path. and Bact.*, 1935, **40**, 147.
11. Maitland, H. B., and Laing, A. W., *Brit. J. Exp. Path.*, 1930, **11**, 119.

12. Steinhardt, E., and Lambert, R. A., *J. Infect. Dis.*, 1914, **14,** 87.
13. Plotz, H., personal communication.
14. Kimura, R., and Fujisawa, Y., *Z. Immunitätsforsch.*, 1932, **74,** 384.
15. Maitland, H. B., Laing, A. W., and Lyth, R., *Brit. J. Exp. Path.*, 1932, **13,** 90.
16. Minerwin, S., and Schmerling, A., *Centr. Bakt., 1. Abt., Orig.*, 1926, **100,** 310.
17. Maitland, H. B., and Maitland, M. C., *Lancet*, 1928, **2,** 596.
18. Carrel, A., and Rivers, T. M., *Compt. rend. Soc. biol.*, 1927, **96,** 848.
19. Eagles, G. H., and McClean, D., *Brit. J. Exp. Path.*, 1929, **10,** 35; 1930, **11,** 337.
20. Craciun, E. C., and Oppenheimer, E. H., *J. Exp. Med.*, 1926, **43,** 815.
21. Hach, I. W., *Centr. Bakt., 1. Abt., Orig.*, 1925, **94,** 270.
22. Steinhardt, E., Israeli, C., and Lambert, R. A., *J. Infect. Dis.*, 1913, **13,** 294.
23. Webster, L. T., and Clow, A. D., *J. Exp. Med.*, 1937, **66,** 125.
24. Carrel, A., *J. Exp. Med.*, 1926, **43,** 647.
25. Rivers, T. M., Haagen, E., and Muckenfuss, R. S., *J. Exp. Med.*, 1929, **50,** 665.
26. Saddington, R. S., *Proc. Soc. Exp. Biol. and Med.*, 1934, **31,** 693.
27. Striegler, E., *Zentr. Bakt., 1. Abt., Orig.*, 1933, **128,** 332.
28. Zinsser, H., and Schoenbach, E. B., *J. Exp. Med.*, 1937, **66,** 207.
29. Fischer, A., Gewebezüchtung, Munich, Verlag Rudolph Müller & Steinicke, 3rd edition, 1930.
30. Hallauer, C., *Z. Hyg. u. Infektionskrankh.*, 1931, **113,** 61.
31. Köbe, K., and Fertig, H., *Zentr. Bakt., 1. Abt., Orig.*, 1936, **138,** 14.
32. Plotz, H., and Ephrussi, B., *Compt. rend. Soc. biol.*, 1933, **112,** 525; **113,** 711.
33. Hallauer, C., Die Züchtung der Virusarten ausserhalb ihrer Wirte. Die Viruszüchtung im Gewebsexplantat, in Doerr, R., and Hallauer, C., Handbuch der Virusforschung, Vienna, Julius Springer, 1938, pt. 1, 379.
34. Parker, R. C., *J. Exp. Med.*, 1936, **64,** 121.
35. Parker, R. C., Methods of tissue culture, New York, Paul B. Hoeber, Inc., 1938.
36. Carrel, A., *Berl. klin. Woch.*, 1913, **50,** 1097.
37. Löwenstädt, H., *Arch. exp. Zellforsch.*, 1925, **1,** 251.
38. Parker, R. C., Methods of tissue culture, New York, Paul B. Hoeber, Inc., 1938, **118.**
39. Gey, G. O., *Am. J. Cancer*, 1933, **17,** 752.
40. Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, **27,** 45.
41. Lewis, W. H., *Carnegie Institution of Washington, Pub. No. 459, Contrib. Embryol.*, 1935, **25,** 161.
42. Gey, G. O., and Bang, F. B., *Bull. Johns Hopkins Hosp.*, 1939, **65,** 393.
43. Ward, H. K., and Tang, F.-F., *J. Exp. Med.*, 1929, **49,** 1.
44. Burnet, F. M., The use of the developing egg in virus research, *Great Britain Med. Research Council, Special Rep. Series, No. 220,* 1936.
45. Robinson, E., Preparation and use of smallpox vaccine, in Virus and rickettsial diseases, Harvard School of Public Health Symposium Volume, Cambridge, Massachusetts, Harvard University Press, 1940.
46. Downie, A. W., *J. Path. and Bact.*, 1939, **48,** 361.
47. Goodpasture, E. W., *Am. J. Path.*, 1925, **1,** 547.
48. Buddingh, G. J., *Am. J. Path.*, 1936, **12,** 511.
49. Shumway, W., Vertebrate embryology, New York, John Wiley and Sons, Inc., 1937.
50. Rhodes, A. J., and van Rooyen, C. E., *J. Path. and Bact.*, 1937, **44,** 357; **45,** 253.

51. Goodpasture, E. W., Woodruff, A. M., and Buddingh, G. J., *Am. J. Path.*, 1932, **8**, 271.
52. Haagen, E., *Zentr. Bakt., 1. Abt., Orig.*, 1931, **120**, 304.
53. Hecke, F., *Zentr. Bakt., 1. Abt., Orig.*, 1932, **126**, 93.
54. Amies, C. R., *Brit. J. Exp. Path.*, 1934, **15**, 180.
55. Plotz, H., *Compt. rend. Soc. biol.*, 1937, **125**, 603, 719.
56. Beard, J. W., and Rous, P., *J. Exp. Med.*, 1938, **67**, 883.
57. Rivers, T. M., Haagen, E., and Muckenfuss, R. S., *J. Exp. Med.*, 1929, **50**, 181.
58. Simms, H. S., and Stillman, N. P., *Arch. Path.*, 1937, **23**, 316.
59. Haagen, E., *Centr. Bakt., 1. Abt., Orig.*, 1928, **109**, 31.
60. Andrewes, C. H., *Proc. Roy. Soc. Med.*, 1939, **33**, 75.
61. Hallauer, C., *Z. Hyg. u. Infektionskrankh.*, 1933, **115**, 616.
62. MacCallum, F. O., *Brit. J. Exp. Path.*, 1936, **17**, 472.
63. Muckenfuss, R. S., and Rivers, T. M., *J. Exp. Med.*, 1930, **51**, 149.
64. Muckenfuss, R. S., *J. Exp. Med.*, 1931, **53**, 377.

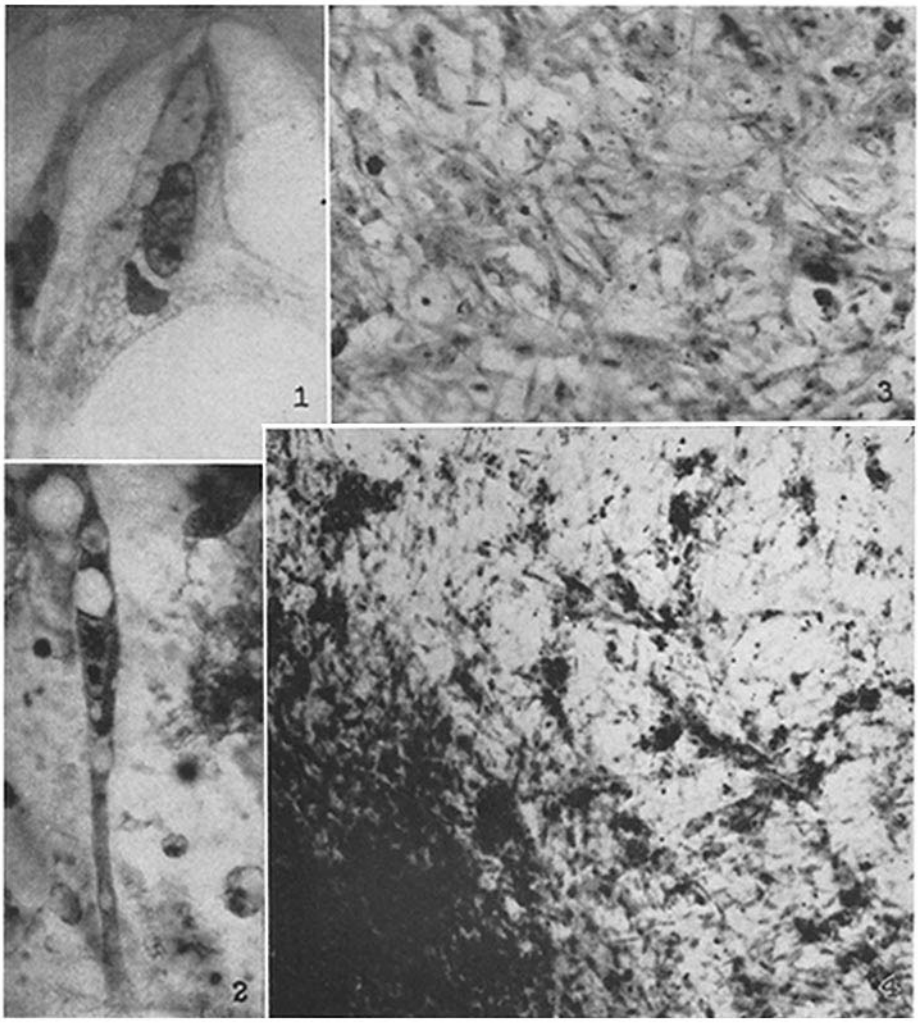
EXPLANATION OF PLATE 17

FIG. 1. A fibroblastic cell from a culture of cardiac muscle of an 11 day chick embryo inoculated 15 days previously with vaccinia virus. A large inclusion is shown directly below the nucleus. The cell was found in the same preparation of which a low power view is shown in Fig. 3. Acid-fuchsin and Loeffler's methylene blue. Approximately × 1330.

FIG. 2. A spindle-shaped cell from the same preparation. Two inclusions are present: one extends from below the nucleus upward along its right border; another lies between the two vacuoles above the nucleus. Acid-fuchsin and Loeffler's methylene blue. Approximately × 1100.

FIG. 3. Low power view of the same preparation which contained the cells shown in Figs. 1 and 2. Note the generally good condition of the cells. Acid-fuchsin and methylene blue. Approximately × 170.

FIG. 4. Low power view of a portion of the cell growth in a culture of embryonic chick tissue infected 24 days previously with vaccinia virus. Large numbers of cells in apparently good condition are to be seen. The increasing density of the plasma at this stage due to "patching" renders it difficult to obtain detail in the photograph. Hematoxylin and eosin. Approximately × 90.



(Feller *et al.*: Vaccinia virus in chick embryonic tissue cultures)