THE INTERACTION OF ROUS SARCOMA VIRUS AND CELLS IN VITRO

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ABSTRACT

By the use of new methods for assaying in vitro Rous sarcoma virus (RSV) and Rous sarcoma cells and for isolating the progeny of single particles of RSV, it has been found that the morphology of Rous sarcoma cells is partially controlled by genetic factors in the virus. The influence of the state of the cell before infection, operating directly on the virus-cell complex has been demonstrated. A single particle of RSV is able to initiate infection, but viral genetic factors and genetic and physiological factors in the cell determine whether or not a cell does become infected. Host range mutants of the virus are described. It was shown that there exists a state in which resistant cells become competent to support the growth of RSV.

RSV rapidly adsorbs and penetrates into cells. After a 12 hour period new virus appears. The final rate of virus release of 10 focus-forming units per cell per 10 hours is reached about 40 hours after infection. The ability of a cell to produce RSV is transmitted to its progeny as an intracellular event, the number of Rous sarcoma cells doubling every 15 - 20 hours. Immediately after infection the X-ray resistance of the capacity of a cell to release virus is the same as its capacity to divide. By 15 - 20 hours after infection, the resistance of the capacity of the cells to release virus increases about 30 times.

Direct observation of isolated cells in microdrops shows that a cell can release virus and then divide. Interference caused by one particle of inactive virus, operating on some intracellular process after penetration of the virus into the cell was found.

The relationship between RSV and temperate bacteriophage, the relevance of the findings to the cancer problem, and the relationship between RSV and other animal viruses are discussed.

INTRODUCTION

General

Most of the basic generalizations now made about viruses derive from work done on bacteriophage. The early recognition of the advantages of the plaque assay and the introduction of the one-step growth curve (Ellis and Delbruck, 1939) led to these advances in understanding. With the introduction of similar techniques for animal viruses by Dulbecco (1952), there has been a rapid increase in knowledge of animal viruses. The results obtained tend to emphasize the similarities of all viruses.

Bacteriophages can be divided into two large classes. The first class is that of virulent phages. After infecting a cell, virulent phages multiply rapidly; a large number of progeny particles are released upon lysis of the cells. The second class consists of the temperate phages. These are capable of forming a stable symbiotic relationship with the infected bacteria. In this state the potentiality to produce mature phage is perpetuated in all bacteria from one generation to the next. Under ordinary conditions, however, only a small fraction of the cells at any given time produce mature phage. As in the case of virulent phage, these cells lyse.

Evidence has been obtained from genetic and radiological experiments indicating that temperate phages can interact in an intimate way with the genetic material of the host bacteria (Bertani,

1958, Stent, 1958). Bacterial crosses made with lysogenic bacteria show that the genetic material of the temporate phage is localized on a specific region of the bacterial chromosome and behaves in crosses as a single bacterial marker. It has also been shown that temperate phages grown in one bacterial strain can carry some of the genetic traits of that strain to a second strain of differing genotype. This process is called transduction and the transduced characters may become permanently established in the new host. Another process superficially similar to transduction involves conversion of the character of an infected bacterium e.g. sero-type (Uetake, et al., 1958), but this transfer is only mediated by viable phage whereas transduction is mediated only by defective phage (Arber, et al., 1957).

The radiological evidence is of a complex nature involving kinetic studies of the sensitivity to various types of radiation of the free virus of the host cells before infection and of the virus-cell complex (Garen and Zinder, 1955). It has served to supplement and extend the genetic evidence (see discussion).

Similarly, animal viruses have been divided into cytocidal and moderate (Dulbecco, 1957). Cytocidal viruses are analogous to virulent phage. They kill the infected cells within a time comparable to the division time of the cell; the genome of the virus does not interact detectably with the host genome. Moderate viruses are analogous to

temperate bacteriophage. By definition, cells infected with a moderate virus survive for a period of time long with respect to the doubling time of the cell, the infected cells divide giving rise to progeny cells which are infected hereditarily and not by reinfection from outside; the viral nucleic acid (i.e. the viral genome) in the cells of the infected clone interacts with the host nucleic acid.

Among the animal viruses an outstanding system for study of the properties of moderate viruses is found in the Rous sarcoma virus and its host cells. The Rous sarcoma virus, (RSV), since its original isolation (Rous, 1911), has been associated with the production of tumors. This association made reasonable the hypothesis that this virus differed from other animal viruses in its interaction with the host cells. It has been established (Rubin and Temin, 1959, Temin and Rubin, 1959) that the Rous sarcoma virus is a moderate virus. Subsequently new evidence has been obtained which strengthens this conclusion. These points will be discussed in this thesis.

The study of the Rous sarcoma virus is interesting from the point of view of oncology as well as virology, since it throws some light on various problems such as the nature of cancer and the relations between viruses and cancer. RSV was originally isolated from a spontaneous sarcoma in a Plymouth Rock hen. In the course of early passage of the virus from one animal to another, variation occurred in the host range of the virus so that it could grow in non-blood

relatives of the animal of origin. Soon thereafter the character of the tumor changed by the appearance of hemorrhagic growths, of spherical rather than spindle shaped cells and of giant cell forms (Rous and Murphy, 1913). Duran-Reynals (1942, 1959) carried out extensive experimental work showing that variation occurred readily in the tumor produced by RSV, especially after passage to another host, usually ducks, followed by a return to fowls. In this way virus causing different types of tumors was isolated. Genetic variation of the virus with respect to host range and with respect to morphological character of the Rous sarcoma cells has now been obtained in vitro. These variations will be discussed in this thesis.

Duran-Reynals also emphasized the importance of the age of the host in determining the nature of the response to infection with RSV, such as an hourly increase in resistance of ducklings to infection (Duran-Reynals, 1946). The influence of the host cell on the consequences of infection with RSV can also be detected in vitro. This phenomenon, described as competence, was discussed by Temin and Rubin (1958). It will also be analyzed in this thesis.

Problems Concerning the Rous Sarcoma Virus Specifically

In a recent article giving a comparative survey of animal viruses, the Rous sarcoma virus is put with a group of viruses causing various neoplastic diseases of fowls having neurolymphomatosis as a prototype virus (Burnet, 1959). It is the consensus of most workers (Andervont, 1959; Beard, 1957; Oberling and Guerin, 1954)

that while each disease of this complex possesses distinct properties, all of the diseases, as well as all of the viruses possess undeniable relationships. The chief evidence for this conclusion is cross-neutralization in serological tests.

The RSV is outstanding as an experimental object among the members of this group in that it can be used satisfactorily for quantitative work of physiological or genetic nature. This is mainly because of efficient assay procedures available for this virus. The customary technique for assaying RSV has been the production of tumors in chickens (Rous, 1911, Bryan, 1946). More recently, the virus has been assayed by infecting the choricallantoic membrance of the developing chicken embryo (Keogh, 1938, Rubin, 1955, Prince, 1957). A great step forward, however, was the development of an *in vitro* technique of assay.

The attempt to cause the change of normal cells to cancer cells by RSV *in vitro* was begun by Carrell (1924). The first clear demonstration of the change was given by Halberstaedter, *et al.* (1941), who exposed chick fibroblast cultures to pieces of intensely irradiated Rous sarcoma cultures and observed changes in the normal cells giving rise to colonies of highly polymorphic cells of characteristic structure and behavior, which liquified the plasma clot with enhanced intensity. This culture was similar to one derived from a chicken sarcoma. Manaker and Groupe (1956) showed that infection and transformation *in vitro* could be used as a means of assaying the virus analogous to

the plaque method for cytocidal viruses. Such a technique was developed and described by Temin and Rubin (1958) and will be discussed below.

Two properties of RSV must be noted. The first is the probable RNA nature of RSV. This conclusion is based on the absence of deoxyribose from partially purified preparations of RSV containing almost no normal tissue or nuclear contaminants (Bather, 1957) and from the observation that an electron-opaque core of the virus disappeared upon treatment with RNA-ase, but not with DNA-ase or trypsin (Epstein, 1958). The second is its lability at 37°. A half-life of 2-1/2 hours at 37° in tissue culture medium was reported by Rubin (1955) and confirmed for the medium used in the work described in this thesis. The virus is 80 mic. in diameter.

Several reports on the growth of RSV have been published. Carr (1953) studied the replication of RSV in the leg muscle of young chicks. He observed a drop in infectivity 16 hours after inoculation, followed by a sudden increase starting at 70 hours. Harris (1954) reported comparable results with RSV on the chorioallantoic membrane of embryonated eggs, as did Groupe and Rauscher (1957) for RSV multiplying in the brain of newborn chicks. Prince (1958) extended Harris' work with the chorioallantoic membrane. He verified the existence of an eclipse period, followed by two successive exponential growth phases, the first rapid, the second slower, following the onset of mesodermal hyperplasia.

Rubin (1955, 1957) made the first studies of RSV growth kinetics in vitro. He cultivated sarcoma cells from one-week old tumors in chicks and verified that virus could be produced by all cells, but was released in a slow trickle. Detailed in vitro studies of the process of infection have now been carried out (Temin and Rubin, 1959) and will be discussed below. After the conclusion of this work a study of RSV growth in vitro using an egg assay appeared (Vigier and Golde, 1959). These workers again confirmed Carr's findings of an eclipse period followed, after two days, by exponential rise in free virus.

PLAN OF THESIS

The methods used will be described. The morphological changes produced in vitro by RSV will then be presented. These changes will be shown to be under the control of factors present in the cell and in the virus. Next will be considered the factors involved in the establishment of infection. It will be shown that whether or not a cell is infected depends on the genome of the virus and on the state of the cell. Several aspects of the growth of RSV in the infected cells, as determined by the appearance of infective virus and by changes in the X-ray resistance of the cell capacity will be presented. Evidence from the growth of infected cells and from single cell experiments will be used to demonstrate the moderate nature of RSV.

The preceding experiments can be looked upon as describing changes mediated by a single particle of virus. Other studies concerning changes mediated by more than one particle infecting the same

cell will be given.

MATERIALS AND METHODS

Solutions and Media.

Growth medium

7 parts Eagle's medium (Eagle, 1955) with double or four-fold concentrations of amino acids and vitamins and 4.2 g $NaHCO_3$ per liter.

2 parts Difco-Bacto-Tryptose phosphate.

1 part serum (chicken or calf).

Agar medium

1 part growth medium

1 part two-fold concentrated Eagle's medium.

1 part 1.8% agar in distilled water.

Trypsin

0.25% Bacto-Trypsin in tris buffer.

TD

0.05 M tris buffer in isotonic sodium and potassium chloride solution, pH F.4 - F.5.

Incubation of cultures. All cultures were made in petri dishes and incubated at 37° in a humidified incubator. The pH was regulated at 7.3 by a bicarbonate buffer controlled by injecting CO_2 into the incubator so as to maintain a constant CO_2 tension in the atmosphere.

Examination of cultures. Cultures were examined at 25 and 100 fold magnifications of a Zeiss plankton (inverted) microscope.

Cultivation of cells. Embryos of two varieties of chicken (White Leghorn and New Hampshire brown) and of ducks were used. Primary cultures of 10 day old embryos were made by trypsinizing the decapitated embryos, according to the method of Dulbecco as modified by Rubin (1957a). For million cells in 10 ml of medium were placed in 100 mm petri dishes. Within a few days rich cultures developed, containing a mixed population of cells with fibroblasts predominating. After three days incubation, secondary cultures were made by trypsinizing the primary cultures. The primary cultures were washed twice with TD and 2 ml of 0.05% trypsin in TD added. After 15 minutes incubation 2 ml of medium was added and the cells concentrated by centrifugation. Two hundred thousand cells in 3 ml of medium were placed in 50 mm petri dishes. After incubation overnight these plates, containing a loose network of spread cells, were used for the assay of Rous sarcoma virus and Rous sarcoma cells. The cultures consisted chiefly of elongated, fibroblast-like cells. The exact nature of these cells is unknown. They will be referred to as fibroblasts according to their morphology.

Primary cultures of heart fibroblasts were made from 16 day embryos by trypsinization, according to a method described by Franklin et al. (1957) for lung cultures. After incubation of the primary culture, secondary cultures of 2×10^5 cells were made as above.

Assay for Rous sarcoma virus (RSV). The nutrient medium was removed from a secondary culture and virus in 0.2 ml of medium added.

After adsorption for 30 minutes at 37° in the CO_2 incubator, 5 ml of agar medium was added. Three and six days later the culture was fed by the addition of 2.5 ml of agar medium on top of the first agar layer. At six and nine days the plate was completely scanned for foci (see below) at a 25-fold magnification of the inverted microscope.

Assay for Rous sarcoma cells. Rous sarcoma cells or Rous cells is a term designating the transformed, virus-releasing cells induced by the Rous sarcoma virus in vitro. To determine the number of Rous sarcoma cells in a mixed cell population, the cells were placed in suspension with trypsin and counted. A suitable aliquot containing about 100 Rous sarcoma cells was added to a secondary culture. After incubation for about 8 hours to allow attachment of the cells to the glass, the supernatant was carefully removed and 5 ml of agar medium was added. The plate was then treated as in the assay for RSV.

Selective assay for virus-producing cells. In the assay for Rous sarcoma cells described in the previous section, a focus could be initiated by division of one of the plated Rous cells or by infection of a cell on the assay plate by virus released from the Rous cell (unless the cells on the assay plate were resistant to the virus). To determine selectively the number of virus-producing cells, infected cells suspended in medium were X-irradiated so they could not divide, but continued to produce virus (see below), and then plated as in the assay for Rous sarcoma cells. No increase in virus release was found after irradiation.

Virus. The virus used in this work was originally obtained in 1957 from Dr. Bryan of the National Cancer Institute. It has been kept in this laboratory by passage in tissue culture. Supernatants of cultures of Rous sarcoma cells were collected and frozen at -60° until used.

Isolation of clonal virus by picking foci. Progeny virus from single particles was obtained by picking the cells from a focus of Rous sarcoma cells developing on an assay plate. After incubation for 9 days, the assay plates were scanned and the position of foci marked on the glass. The agar was removed, the plate washed twice with TD and a 0.0125% solution of trypsin in TD added. Under the high power of a dissecting microscope the cells of individual foci were sucked into a capillary pipette as they came loose from the glass. The cells were then transferred to drops of medium under paraffin oil (Lwoff et al., 1955). After incubation for 6 hours to 2 days, the supernatants of the drops were removed to 1 ml of medium in small test tubes and the solutions frozen. There were between 0 and 104 focus forming units (FFU) of RSV recovered per drop, depending on the number of cells successfully transferred. In most experiments, all foci picked produced large amounts of virus. Virus was not found only when the cells in the drop were few in number or had not survived transfer.

Cloning of cells. Colonies of cells derived from single cells were obtained by the techniques of Puck and Marcus (1955), which consists in plating dilute suspensions of cells on an X-irradiated layer of cells called the feeder layer. Feeder layers of chick or duck embryo cells

were made by irradiating with 4000r cultures containing 2 to 6 x 10^5 cells in a 50 mm petri dish. Dilute suspensions of the cells to be cloned in 2 ml of medium were added to the petri dish and allowed to settle and to adhere to the glass. Four ml of agar medium was then added to the supernatant fluid. After 7 days of incubation, the clones arising from the non-irradiated cells could be distinguished, the irradiated cells survived but did not multiply.

X-irradiation. Cells and virus were X-irradiated with a Machlett OEG-60 tube with beryllium end window, operated at 50 kilovolts and 30 milliamperes. When high doses of X-rays were required no filter was employed and the material was irradiated in open petri dishes 5.0 cm from the X-ray target. For lower doses, an aluminum filter 0.38 mm in thickness was used. Cells were irradiated with low doses in petri dishes covered by their usual glass cover at 6.6 cm from the X-ray target. The dose of irradiation was 2640r per minute at the low doses, 20,000r per minute at the higher doses.

Assay for cell-associated virus. In order to determine the amount of infective virus associated with cells, either intracellular or stuck on the surface in a dilution resistant form, 5 x 10^5 cells in 1 ml of medium were frozen and thawed 3 times using a dry-ice-acetone mixture and a 37° water bath. Two-tenths of an ml of a suitable dilution was assayed for RSV.

Drop cultures. Drops of 0.01 ml of medium were placed in a 50 mm petri dish having a ruled grid on the bottom under 15 ml of paraffin

oil (Lwoff et al., 1955; Vogt and Dulbecco, 1957). Cells were placed in the drops with a micropipette using the high power of a dissecting microscope.

Neutralizing serum. Chicken serum capable of neutralizing at a one to five dilution 99% of 100 FFU in 30 minutes at 37° was obtained from Dr. Bryan. It had a K of 5 to 10. It was obtained as convalescent serum from a chicken surviving infection with RSV.

Ultraviolet irradiation. Ultraviolet irradiation was given from a Westinghouse germicidal lamp. The virus was in 1 ml of medium, 11-3/4 inches from the lamp.

RESULTS

The Control of Cellular Morphology

Morphological changes in chick embryo cells following infection with wild-type RSV. One or two days after addition of RSV of the usual laboratory stock to a secondary culture of chicken embryo fibroblasts, rounded refractile cells appear. After 3 or 4 days, groups of such cells -- foci -- can easily be distinguished from the background fibroblasts (Photo 1, 2, 3). The number of foci is proportional to the dilution of virus innoculated, no foci appear if virus is treated with neutralizing serum before infection, and virus can be obtained from the cells in a focus. Therefore, the cells in the focus are called Rous sarcoma cells and are considered to have been induced in the culture by RSV. All virus producing, morphologically altered cells are called Rous Sarcoma cells. The virus producing this particular type of focus is designated morph+ and is considered to have the wild-type character for morphology.

The rounded refractile cells differ markedly in their colonial characteristics from the normal fibroblasts. They grow in grape-like clusters only loosely attached to the glass. Often a focus becomes multilayered and the round cells migrate out on top of the other cells (Photo 2).

The relative growth rates of the Rous sarcoma cells in a focus and the rest of the cells on an assay plate are compared in Fig. 1. Rous sarcoma cells were added to an assay plate and every day the number of

cells in 10 - 20 foci were counted. At the same time the total number of cells on a plate was determined. The initial growth rates of the normal cells and the Rous cells are similar, the number of cells doubling every twenty hours. However, the Rous cells continue to increase in number even though the growth rate of the normal cells has greatly decreased.

This continued growth of Rous sarcoma cells after normal cells have stopped seems to occur even in cultures where the majority of cells have been transformed. As shown in Table 1, the number of cells at 6 days in a culture of uninfected fibroblasts and in a culture of fibroblasts infected with a multiplicity of over 1 of RSV, was strikingly different.

Morphological changes in chick embryo cells following infection with morph^{f} RSV. A second type of RSV producing a different type of focus was isolated with a frequency of 10^{-3} - 10^{-4} as a mutant of morph+. It was also isolated from foci of fusiform cells produced by stock Bryan RSV. This virus, designated morph^{f}, produces foci of long, narrow fusiform cells (Photo 5).

Role of the virus in determining the morphology of Rous sarcoma cells. The production of different morphological types of foci, described above, could be due to two different mechanisms: a) the virus could cause a direct transformation of the cell; b) some kind of selective mechanism could exist so that different virus mutants would only affect different pre-existing cell types. The second hypothesis is rendered unlikely by the following experiment. Two clones of chick fibroblasts were isolated.

After each clone had reached sufficient size, assay plates of 2×10^5 of its cells were made in 50 mm petri dishes. Virus $H1C^4$, producing foci of long fusiform cells, and a mutant from it, H1C413, producing foci of round refractile cells, were assayed on these plates. As seen in Table 2 and Photos 5 and 6, virus H1C4 produced foci of long fusiform cells and virus H1C413 produced foci of round refractile cells on cells derived from the same clone. The reason for the variation in number of foci on the two lines of cells will become clear later.

It appears, therefore, that the virus, according to its genotype, determines the morphology of the infected cell.

Morphological changes in chick heart fibroblast following infection with RSV. Foci produced by RSV ($morph^f$) on chick heart fibroblast resemble those formed by this virus on chick embryo fibroblast. On the contrary, the foci produced by wild-type RSV ($morph^+$) on heart fibroblasts are sometimes like those formed on secondary fibroblasts, but mostly are composed of pale, separated cells. The different types of foci produced by the same virus on two different types of cells could be due to genetic differences between the cells (as has been found with duck cells, see below) or to differences between heart and other fibroblasts. To clarify this point cultures of heart fibroblasts and of body wall fibroblasts were made from a single embryo. The foci of $morph^+$ virus on heart fibroblasts were of pale, separated cells, whereas those on body fibroblasts were round refractile cells. The previous differentiation undergone by a cell affects the morphology of the Rous sarcoma cell.

TABLE 1

GROWTH OF FIBROBLASTS AND RSV INFECTED FIBROBLASTS

Virus	Initial Cell Number	Cell Number at Six Days
--	2×10^5	7.8×10^5
--	2×10^5	7.3×10^5
H1D1D1(morph$^+$)	2×10^5	40×10^5
26B4(morph^{26})	2×10^5	30×10^5

Parallel cultures of heart fibroblasts were made. Two were infected with a multiplicity of virus over one, two were not. Agar was added to all cultures. At six days the agar was removed and the number of cells counted, after removing them from the glass with trypsin.

TABLE 2

ROLE OF VIRUS IN DETERMINING THE MORPHOLOGY OF ROUS SARCOMA CELLS

Virus stock	Type of foci produced on clonal fibroblasts	
	a	b
HlC4	5 fusiform	ca. 1000 fusiform
HlC413	5 round	ca. 1000 round

Assay plates of 2×10^5 cells were made from two separately isolated lines of clonal fibroblasts (a and b). Two virus stocks producing different types of foci on secondary cultures of chick embryo fibroblasts were assayed on these plates. The type of focus produced is given.

Morphological changes in duck embryo cells following infection with RSV. In addition to the difference in morphology of foci produced on genetically similar cells from different organs, there is a difference in the foci produced on genetically different cells. Three or four days after infection of secondary duck embryo cultures with RSV morph^{+} thick refractile fibroblasts appear. These grow to form piles of cells (Photo 7) having a pronounced brown color due to increased granularity of the cells. Foci appearing following infection with RSV morph^{f} are composed of thinner fusiform cells which do not make piles.

Role of the uninfected cell of the assay layer in determining the morphology of Rous sarcoma cells. The last two sections have shown that the morphology of Rous cells is affected by the cell infected, as well as by the viral genome. The following experiments were carried out to determine whether or not this influence of the cell was of a physiological nature, as one of feeding originating in the uninfected cells surrounding the transformed cell. Duck and chick cells were infected with virus of the same morphological type. The infected cells of each type were then cloned on X-rayed feeder layers of duck or chick cells. The resulting clones of Rous sarcoma cells were morphologically typical of the cell of origin and not of the feeder layer.

To test whether feeder layers of unirradiated cells would give a similar result, heart fibroblasts from embryos resistant to the usual lines of RSV (see below) (designated as h^{+}, i.e. virus with host range character of wild-type) were chosen as a feeder layer.

These cells are sensitive to the host range mutant of RSV h^c. The experiment consisted of two parts. In the first, resistant heart fibroblasts were infected with h^c (see below) virus. The infected cells were plated in parallel on cultures of chick embryo fibroblasts and on cultures of heart fibroblasts. The morphological types of the foci produced were typical of the respective feeder layers (Table 3). The foci in these cultures contained cells from the feeder layer which had become infected by virus released from the infected heart fibroblasts. This was confirmed by the observation that X-raying the infected heart fibroblasts so they were unable to divide before plating them did not reduce appreciably the number of foci produced. In the second part of the experiment regular chick fibroblasts were infected with h^+ virus and the infected cells were plated in parallel on sensitive fibroblasts and on resistant heart fibroblasts. One type of focus was produced on both cell layers (Table 3). This shows that the cells of the feeder layer do not affect the morphology of cells that divide on them. If, however, the cells of the feeder layer become infected, they give rise to Rous sarcoma cells according to the nature of the feeder cells.

From the foregoing experiments it may be concluded that the virus and the host cell together determine the morphology of the Rous sarcoma cell.

TABLE 3

Cell of origin	Infecting virus	Heart fibroblasts	Chick embryo fibroblasts
Heart fibroblast	H1D1 (h^c $morph^+$)	347 foci of pale separated cells 3 round	1000 foci of round cells
Chick embryo fibroblast	H3E1 (h^+ $morph^{r3}$)	500 foci of round cells	500 foci of round cells

Resistant heart fibroblasts were infected with host range virus. The infected cells were plated in parallel on cultures of chick embryo fibroblasts and on cultures of the resistant cells. At the same time regular chick fibroblasts were infected with a virus which produced foci on them and no foci on the resistant cells. The infected cells were plated as above. The types of foci produced are recorded.

THE INFECTION PROCESS

Number of particles required to initiate infection with RSV.

It has been characteristic of virus systems to find a linear titration curve. This has been shown to mean a single particle is adequate to initiate infection (Dulbecco and Vogt, 1954). Similar results have been found for RSV using in vivo assay techniques (Keogh, 1938; Rubin, 1955; Prince, 1957). This linear dose response has been confirmed in vitro by the following experiment. A series of two-fold dilutions of virus were assayed on duplicate plates and the number of foci per plate plotted against the relative virus concentration. The results of the experiment are reported in Fig. 23. A slope of 1 indicates that a single particle is adequate to initiate focus formation.

Variation in efficiency of plating (eop) of RSV. As seen in Table 4, the efficiency of plating of virus varied with the type of cell used for assay. Virus having a higher plating efficiency than wild-type on resistant chick cells was isolated from foci appearing when virus from a clonal line of morph$^+$ Rous cells was assayed on the resistant cells. Virus having a higher eop on duck cells was isolated from a single focus after three passages of Bryan RSV from old Rous cells on duck cells. This virus, designated as h^d, was then found to have a higher eop than h^c virus on resistant chick cells. h^c virus with a higher eop on duck cells has been isolated as mutants from h^c stock.

Isolation of non-host range virus from foci on resistant cells. The plating of h^+ virus on resistant cells with an efficiency of one in a

thousand could be due to the presence of this proportion of mutant h^c virus particles. To test this possibility, virus was isolated from foci produced by h^+ virus on resistant chick cells. As seen in Table 5, the virus isolated from these foci was not mutant, but retained the h^+ character of the original virus. Similarly, virus isolated from the foci on duck cells infected with h^+ or h^c virus, corresponded in type to the original virus. Even after several passages on the resistant cells the virus was still found to have the original host range character. Rarely, ca. in 1/100 of the foci, the isolated virus was found to be a host-range mutant of the original virus and to plate with higher efficiency on resistant cells, as for instance, in the last experiment in Table 5. Therefore, mutation of the host range character can occur. But, because of the frequent occurrence of foci with virus of parental host range, it has not been possible to use increased host range as a selective marker.

Effect of virus multiplicity on the number of foci appearing on resistant cells. The reduced efficiency of plating of h^+ virus on resistant cells could be due to a decreased probability of infection by each virus particle or could be due to the existence of two classes of cells, resistant and sensitive, in the resistant population of cells. A study of the titration curve of h^+ virus on resistant cells -- where the proportion of virus infected cells is determined at various virus concentrations -- enables a choice to be made between these two possibilities. Under the first hypothesis the number of foci should continually increase with increasing multiplicity; under the second hypothesis the titration curves should

TABLE 4

VARIATION OF EFFICIENCY OF PLATING OF RSV

Cell	Virus		
	h^+	h^c	h^d
Sensitive chick embryo fibroblast	1	1	1
Resistant chick embryo fibroblast	10^{-3}	.1-.3	.1-.3
Duck embryo cell	10^{-7}	10^{-3}-10^{-4}	.01-.5

Secondary plates of 2×10^5 cells were made from sensitive and resistant chicken embryos and from duck embryos; various clonal stocks of virus were assayed in the usual manner. The relative titres were referred to that on sensitive chick cells.

TABLE 5

ISOLATION OF VIRUS FROM FOCI ON RESISTANT CELLS

Original Virus		Isolated from foci on	Character of isolated virus
h^+	H3C1	Resistant chick	h^+
h^+	H5E1	"	h^+
h^+	H5E5	"	h^+
h^+	H5D3	"	h^+
h^+	28A3	Duck	h^+
h^c	12D1	"	h^c
h^+	H51	Resistant chick	h^c

Virus was plated on resistant cells. The few foci that appeared were picked, virus obtained and replated on resistant cells and sensitive cells as a control. The character of the recovered virus is given.

break and become horizontal when all the sensitive cells are infected.

That the second hypothesis is correct is shown by the following experiment (Table 6). Ten-fold dilutions of an h^+ virus titring 5×10^6 FFU/ml on sensitive cells were assayed on cultures of resistant cells. The number of foci per plate increased ten times with successive higher virus concentration until the last plate when less than a two-fold increase was found. Similar results were found with h^d virus plating with 1% eop on duck cells. An exactly similar curve is found with h^+ virus on sensitive cells; though the plateau level is 1-100% (Fig. 3) and with h^c virus on resistant chick cells; and with h^d on duck cells - suggesting that similar resistance phenomena are operative in all cases.

Occurrence of sensitive cells in clonal populations of resistant cells. These results indicate that a population of resistant cells contains some sensitive cells. Since the experiment reported in Table 6 was done with cells from a single embryo, the sensitive cells must have appeared during development of the embryo. To see if this change occurs during multiplication of the cells in vitro, a population containing a majority of resistant cells, and about 0.01% sensitive cells was cloned on several petri dishes so that five hundred clones appeared per plate. When the total number of cells reached 10^5 per plate, the cells were trypsinized and one assay plate was made from each of the original plates. The assay plates were infected with wild-type virus at a multiplicity greater than 1 FFU/cell. The number of foci developing per plate is given in Table 7. There were about five foci per plate. Since each assay plate

contained cells derived from about 500 clones -- with about 100 cells per clone -- no clone could have been composed entirely of sensitive cells. Therefore, the sensitive cells must have arisen during the growth of the clones. This experiment can also test the clonality of the change from resistance to sensitivity, according to the principle of the fluctuation test used for microbial mutations by Luria and Delbruck (1942). If the sensitive cells present on the assay plate were the descendants of some variants produced during the development of the clones, their distribution on the assay plates should be of the clonal type, i.e., with a variance much greater than the mean. The observed distribution (Table 7) is poissonian, since the variance (4.75) approximates the mean (4.46). This distribution indicates a non-mutational origin of the change, if the growth rate of the changed cells is not greatly different from that of the other cells.

Frequency of change from resistance to sensitivity. The number of sensitive cells in a resistant population will be a measure of the frequency of change if there are no selective factors operating against the sensitive cells. To remove the possibility of selection, the following type of experiment was done. A population of resistant cells was infected with virus so that all sensitive cells were infected. A few hours later the culture was reinfected. Any new sensitive cells would give rise to foci. This experiment was first performed as the previous experiment on the origin of sensitive cells. Resistant cells with 50 sensitive cells out of 2×10^5 were cloned so that there were 400 clones per plate. After six days the clones were infected on the plate. Six hours later half of the

plates were reinfected. The number of foci on the reinfected plates was twice that on the plates that were infected once (Table 8). (The frequency of sensitive cells in parallel cultures is relatively constant over a long period of time). Both the experiments reported in Table 7 and 8 are inconsistent with the hypothesis of mutation from resistance to sensitivity and growth of the mutant cells. They are consistent either with a transient physiological change (the temporarily sensitive cell would become resistant again later) or with a genetic change (cell would remain sensitive) with little or no multiplication of the mutated cell before infection. However, since the infected cell must multiply to form a focus and since there are completely sensitive cultures, sensitive cells must be able to divide.

In conclusion, concerning the problem of the origin of the sensitive cells in the resistant cultures, it can be said that a resistant cell may change into a sensitive cell, that the resistance of a cell is relative to the genotype of virus infecting it, and the frequency of the change depends on the genotype of the cell.

TABLE 6

TITRATION OF h^+ VIRUS ON RESISTANT CELLS

Virus dilution	1/1000	1/100	1/10	1/1
Number of foci	1	18	195	350

Successive ten-fold dilutions of an h^+ virus stock titering 5×10^6 FFU/ml were assayed on cultures from a single resistant chick embryo. The number of foci appearing was recorded.

TABLE 7

OCCURRENCE OF SENSITIVE CELLS IN RESISTANT CLONES

Plate	a	b	c	d	e	f	g
Number of foci	5	6	9	4	2	5	2
Plate	i	j	k	l	n	o	
Number of foci	5	3	3	3	8	3	

$$\text{Mean} = \frac{58}{13} = 4.46 \qquad \text{Variance} = \frac{57}{12} = 4.75$$

Cells from resistant embryos ($1/10^4$ sensitive cells) were cloned. After two weeks incubation 13 plates with 400 clones each were trypsinized and replated. Virus was then assayed at a multiplicity greater than 1 FFU/cell. The number of foci per plate is given.

TABLE 8

CHANGE IN NUMBER OF SENSITIVE CELLS APPEARING IN RESISTANT CLONES WITH TIME

	Infected once	Infected twice
Number of foci	24, 17, 12	49, 48, 41

Resistant cells with 50 sensitive cells out of 2×10^5 were cloned. Ten days later six plates with 400 clones each were infected. Three of the plates were reinfected six hours later. The number of foci appearing are given.

ENTRANCE OF VIRUS INTO AND INFECTION OF A COMPETENT CELL

Kinetics of adsorption. The first contact between viruses and their host cells is considered to be an adsorption process depending upon the establishment of electro-valent bonds. Adsorption of RSV to sensitive secondary chick fibroblasts was studied in the following experiment. An inoculum of virus containing approximately 50 FFU in 0.2 ml was added to each of several cultures. After adsorption for varying lengths of time, the supernatant was removed and an agar overlay was added. The number of foci per plate is plotted as a function of the time of adsorption in Fig. 4.

Kinetics of penetration. After adsorption, a virus enters the cell. The process of entry can be defined operationally as the loss of sensitivity to antiviral antibody. The entrance process can therefore be followed by determining the degree of protection of the virus from the action of neutralizing serum. Entry can also be defined as loss of infectivity of the adsorbed virus particle. The latter process can be followed by measuring the amount of infectivity remaining after disrupting the cells to release adsorbed virus. The two types of measurements give similar results, indicating that the process defined in the two ways is probably the same.

Several cultures were infected at a computed multiplicity of 4 FFU/cell. After adsorption for 30 minutes, the supernatant was removed and the cultures washed twice. At intervals thereafter, the cultures were tested for the number of foci which were resistant to the

action of neutralizing serum. Two-tenths milliliter of a 1/5 dilution of antiviral serum was added to the cultures for one-half hour, after which the serum was removed and agar added. In parallel cultures the cells were tested for the amount of infective virus adsorbed to the cells. The cultures were trypsinized, the cells disrupted by freezing and thawing three times, and the resulting suspension assayed for RSV. The results are given in Fig. 5. The results indicate that penetration is an exponential process with a rate constant of the order of $1/50\ min^{-1}$.

Intracellular growth of RSV and growth of Rous sarcoma cells. Once the virus has penetrated, no infective virus can be recovered for some time by disrupting the infected cells. After this eclipse period, virus starts to appear as cell associated virus and to be released into the medium. These processes were studied by determining a growth curve of the virus. At the same time the change in number of Rous sarcoma cells was also studied. A number of secondary cultures of chick embryo cells were infected. After adsorption of the virus, they were washed twice with medium and 5 ml of medium was added to each culture. Every 4 hours the supernatant was removed from one or two cultures and 1 ml of fresh medium added. After incubation for 1 hour, the medium was removed and assayed for RSV. Thus, the amount of infectious RSV produced per hour was determined. This technique was used in preference to attempting to measure the total value of virus released at a certain time, because of the high thermal inactivation of RSV at 37°. After removal of the medium, the cells were placed in suspension with trypsin

and assayed for the number of Rous sarcoma cells by plating on a secondary culture. The remaining cells were placed in 1 ml of medium, disrupted by freezing and thawing three times, and assayed for cell associated RSV.

The results are presented in Fig. 6. They show that progeny of the infecting particles appear within the cells and in the medium at 12 - 14 hours. The amount of cell-associated virus and the rate of release of virus increase rapidly from 12 to 24 hours. The curves then break. Their slopes gradually decrease to reach a rate of release of 1 FFU per cell per hour.

The number of Rous sarcoma cells increases more than 50% before any new infectious virus appears, and doubles before an average of one infectious unit of virus has been released per originally infected cell. This increase supports the idea that the ability of an infected cell to produce virus is transmitted to its progeny as an intracellular event. In fact, the increase in number of Rous sarcoma cells in the initial period after infection cannot be due to secondary infection of normal cells by virus released from the cells infected at time zero.

The capacity of the cells to support virus multiplication. The ability of a cell to produce virus can be destroyed by radiation. Survival curves of this ability were determined before and at various times after infection (Luria and Latarjet, 1946). These curves give further information about the intracellular events associated with the multiplication of the virus. Sets of several curves were obtained in the following experiment.

A number of secondary cultures infected with RSV. At zero time and at intervals thereafter cells were placed in suspension, counted and irradiated with successive doses of X-rays. After each dose an aliquot of cells was removed and assayed to determine the number of virus producing cells. Typical results are presented in Fig. 7. Before infection or soon after infection, the ability of the cells to release virus is destroyed with the same X-ray dose as that required to halt cell division. This ability is much more sensitive (30 times) to inactivation than is the free virus. By 15 - 20 hours after infection the ability of the cells to release virus is quite resistant, being about 80% of the resistance of the free virus. The length of time necessary for a cell to develop the more resistant capacity appears to depend on the multiplicity of infection (see below).

This increased resistance of the capacity does not seem to be due to cell-associated infectious virus or to an intracellular pool of virus precursors, i.e., virus genomes with incomplete or no protein coat. As seen in Table 9, Rous sarcoma cells X-rayed so that they had lost the ability to divide continued to release infectious virus even 10 days after irradiation. Any progeny virus present at the time of irradiation would presumably have been matured and released in this time, since it seems unlikely that the intracellular virus pool should contain 10 times the amount of virus released by the cell in one day.

Nature of the virus-cell complex. The data given in the last two sections indicate that after a period of about 20 hours after infection, the infected cells reach some final state. The number of Rous sarcoma cells and the number of virus-producing cells is identical at this time. As seen in Fig. 7, doses of X-ray which prevent division in 99% of the cells have no effect on the number of foci formed by cells plated 25 hours after infection. Since the data in Fig. 6 and the cloning experiments suggest all Rous sarcoma cells divide, the hypothesis was made that production and release of virus is not incompatible with further division of that cell.

Direct evidence for this hypothesis was obtained by the following type of experiment. A suspension of Rous sarcoma cells, derived from chick embryo cells infected in vitro, was washed and diluted. One or two cells were placed in individual microdrops of growth medium. After incubation for 14 hours, the cells were counted and the supernatant of the drop assayed for RSV. Medium was replaced in the drop and it was observed for several days. In some cases the drop was reassayed for RSV. This experiment was carried out several times. All possible combinations of events occurred: virus release and cell division; no virus release and cell division; virus release and no cell division; and no virus release and no cell division. In Table 10 are listed results from eleven cells which were definitely shown to have released virus and then divided. These cells must have produced the virus after being in the microdrop, for after freezing-thawing at zero hours there was less than 1 FFU per

cell and by 14 hours only 2% of these infectious particles would survive thermal inactivation (Rubin, 1955; Temin, unpublished). From 1 to 6 FFU were released by the cells and they underwent one or two divisions after releasing virus. The failure of most cells to divide is consistent with the failure of normal chick embryo cells to divide under these conditions. The failure to demonstrate that all cells release infectious virus is not surprising since the rate of release is only 1 FFU per cell per 5 hours and the half-life of RSV at 37° is 2-1/2 hours.

TABLE 9

PERSISTENCE OF THE ABILITY OF IRRADIATED ROUS SARCOMA CELLS TO FORM FOCI

	Non-irradiated		Irradiated	
	3 days	10 days	3 days	10 days
Number of foci	115,165	-	67	-
	-	198,220	-	102,150

In each two different experiments are shown. Parallel cultures of Rous sarcoma cells were made. One was irradiated with 2,000r. The other was kept as a control. At three or at ten days, both cultures were plated for focus-forming cells.

TABLE 10

VIRUS RELEASE AND CELL DIVISION[a]

Experiment	Number of cells placed in drop	Number of cells after 5 days	Amount of RSV in drop at	
			14 hr	21 hr
1	1	2	1	
2	1	2	2	2
	1	2[b]	4	0
	2	4[b]	3	1
3	1	3	5	
	1	2	1	
4	2	8[b]	2	
5	1	2	3	
6	1	2	2	3
	1	2	6	0
	1	2	1	0

[a]One or two Rous sarcoma cells were placed in individual microdrops of growth medium after the cells had been washed free of extracellular virus. After the cells had spread on the glass they were counted and the supernatant of the drop assayed for RSV. Medium was replaced over the cells and the cell was observed daily for several days. In some cases the drop was reassayed for RSV.

[b]Both cells divided.

INTERACTIONS BETWEEN VIRUS PARTICLES INFECTING THE SAME CELL

The previous data can be looked upon as concerning the interaction of a single particle of RSV with a single cell, and the transformation of that cell into a Rous sarcoma cell. Some additional phenomena occurring when several particles of RSV infect a single cell have been found.

Decrease in time at which the ability of a cell to release virus becomes resistant with increasing multiplicity. The time at which the ability of a cell to produce RSV becomes radioresistant appears to be a function of how many particles of RSV infect a cell. Parallel secondary cultures were infected with RSV at multiplicities of over 3 or less than 1 FFU per cell. After adsorption for 30 minutes, the unadsorbed virus was removed and 3 ml of medium added. At 7, 8, or 11 hours the cells were placed in suspension with trypsin, counted, irradiated with X-rays and aliquots plated for infective centers. Curves were plotted as in Fig. 7, the intersections with the ordinate of the straight line asymptote of the resistant part of the curves used as a measure of the fraction of cells having the more resistant capacity. As seen in Table 11, increasing the multiplicity of infection consistently increases the fraction of cells whose ability to release RSV is more radio-resistant in a fixed period of time.

Interference. A second phenomenon is the prevention of infection by

TABLE 11

DECREASE IN TIME AT WHICH CAPACITY BECOMES RESISTANT WITH INCREASING MULTIPLICITY[a]

Experiment	Multiplicity	Hours after infection when cells irradiated	Per cent of Rous sarcoma cells having resistant capacity
1	3.6	7	22
	0.18	7	8
2	3	8	65
	0.2	8	18
3	3.25	8	45
	0.13	8	12
4	8.85	11	100
	0.59	11	70

[a]Parallel secondary cultures were infected with undiluted and 1:15 or 1:20 diluted RSV stock containing 5×10^5 FFU/ml. After adsorption for 30 minutes the unadsorbed virus was removed and 3 ml of growth medium added. At 7, 8, or 11 hours after infection the cells were placed in suspension with trypsin, counted, irradiated with X-rays, and aliquots plated for infective centers. Capacity curves were plotted as in Fig. F and the back extrapolate of the resistant part of the curve used as a measure of the percentage of cells having the more resistant capacity.

the addition of RSV whose infectivity has been destroyed. This phenomenon is called interference.

Number of particles necessary. A number of secondary cultures were exposed to various concentrations of RSV inactivated by 15 minutes irradiation with UV or incubation at 37° for four days. After 30 minutes the supernatant was removed and the cells superinfected with about 500 FFU of RSV. Thirty minutes later 4 ml of agar medium was added. The proportion of foci appearing on these cultures referred to cultures not exposed to the inactivated virus are plotted in Fig. 8. The data are taken from several experiments. The data fit a simple exponential curve without a shoulder, indicating that a single particle mediates interference.

Blocking of interference by virus neutralizing serum. Mixtures of interfering virus and medium were incubated at 37° for 30 minutes. Parallel secondary cultures were treated with 0.2 ml of these mixtures or of medium. Thirty minutes later the supernatant was removed and 100 FFU of RSV added to each culture. The results in Table 12 show that the treatment of interfering virus with neutralizing serum abolished the interfering activity. This result establishes that the interfering ability is a property of the inactivated virus particle.

Intracellular nature of interference. In order to find out at which step in the virus growth cycle the interfering virus acted, adsorption, penetration or intracellular growth, the following experiment was done. Cultures were infected with 100 FFU of RSV. At various times thereafter

interfering virus was added. Medium was used as a control. As shown in Table 13, virus added 2 hours or more after infection could still interfere with 30% of the previously infected cells even though over 95% of virus has penetrated by this time (see above). Thus interference seems to affect some intracellular process in RSV growth.

TABLE 12

BLOCKING OF INTERFERENCE BY NEUTRALIZING SERUM

Mixture in tubes used to pretreat cultures		Number of foci after virus challenge	
		Exp. (1)	Exp. (2)
0.4 ml	0.1 ml		
Heated virus	Medium	61, 39	56, 70
Medium	Medium	133, 129	109, 153
Heated virus	Serum	114, 165	123, 135

Mixtures of heat inactivated virus with serum and with medium, and medium alone were incubated for 30 minutes at 37°. The mixture was then plated on two assay plates. Thirty minutes later the supernatant was removed and virus added. The results of two experiments are given.

TABLE 13

TIME AFTER INFECTION AT WHICH INTERFERENCE CAN OCCUR

Hours after infection	1/2	1	2	3	4	6	8	10	12
% foci surviving									
Exp. 1	31	30	73		60	71	56	60	67
Exp. 2	31	42	67	61					

A number of secondary cultures were infected with 100 FFU of RSV. After 30 minutes adsorption the supernatant was removed and medium added. At various times thereafter the medium was removed and interfering virus in 0.2 ml added. Medium was used as a control. The number of foci appearing on the two plates was recorded. The data are presented as per cent foci surviving. Two experiments are shown.

DISCUSSION

The data presented in this thesis have bearing on several problems as indicated before. The significance for problems of general virology, oncology, and of the nature and properties of the Rous sarcoma virus as compared to other animal viruses will be discussed.

Relevance of the experiments to general virological problems. The experiments described above, especially the microdrop experiment, show unequivocally that chick fibroblasts infected in vitro with RSV are able to divide and continue to divide for many months while producing virus at a fairly uniform rate. RSV is therefore unique among the animal viruses thus far studied in vitro, since all others, including the related tumor virus of chicken lymphoma (Stoker, 1959), inhibit cell division after infection and ultimately cause cell death. These results and the evidence presented for intracellular transmission of the ability to produce RSV establish RSV as a member, at present the sole member, of the class of moderate animal viruses.

The work presented here on the control of cellular form by the virus illustrates one more important characteristic of this virus. Mutational differences in the virus determine the various forms of the infected cell. This viral action can be designated as conversion of the cell, as discussed in the introduction. Occurrence of conversion with RSV adds another similarity to the properties of moderate animal viruses and temperate phage.

Another striking similarity is seen in the results of the radiobiological experiments reported here particularly if these are compared with similar experiments done with cytocidal viruses (Rubin and Temin, 1959). The extreme sensitivity of the capacity of chick embryo cells to produce RSV is strikingly different from the resistance of the capacity to produce a cytocidal virus (Newcastle disease virus, for instance). This finding is analogous to the observation that the capacity of bacteria for temperate phage growth is much more radio-sensitive than their capacity to support virulent phage growth (Stent, 1958). Stent has suggested that the genome of temperate phage may have to interact with a homologous region of a relatively intact genome of the cell before these phages can multiply, while the multiplication of virulent phage would be essentially independent of the genetic intactness of the host cell.

It has also been found that, relative to the inactivation with X-rays, temperate phage are more resistant to inactivation with ultraviolet light than virulent phage. It has been suggested that the high resistance to UV light is due to the repair of photochemical lesions in the virus by interaction with a homologous region in the genome of the host cell (Garen and Zinder, 1955). Similar pattern of relative UV resistance has been found with RSV when compared to NDV (Sturm, et al., 1932; Rubin and Temin, 1959). The parallelism of the radiobiological results for RSV and temperate phage strongly suggests that RSV also interacts with the genome of the host cell. As yet, no direct evidence for interaction

with the host genome has been found for RSV. Duran-Reynals interpreted his results (1959) to show a host effect on the virus analogous to the host-dependent induction of mutants seen in lambda (Weigle, 1953) and T3 (Fraser, 1957). The experiments of Gye and Purdy (1933) and Harris (1956) have been interpreted by Dulbecco (1957) as indicating transduction of host antigens by RSV. Experiments now in progress may give more definite evidence of this nature.

There are differences between RSV and temperate phage. One is the continued liberation of RSV from an infected cell. Infective phages are only liberated from a lysogenic bacterium upon its lysis. This difference may merely be due to the difference in cell envelopes between animal and bacterial cells. A second difference is the occurrence of a lytic response in a proportion of cases when a temperate phage infects sensitive cells and the absence of such a response with RSV in vitro under the conditions so far explored. Hemorrhagic blebs in vivo may indicate a cytocidal action of the virus on some cells, but may also be due to a secondary effect, similar to the killing of Rous sarcoma cells in vitro by their own toxic products.

Relevance to the problem of the role of viruses in the genesis of cancer. The variation previously observed in the morphology of tumors induced by RSV and in the host range of RSV (see introduction) can now be partly explained. The existence of mutations occurring in the virus for factors controlling the morphology of the transformed cell and for host

range of the virus has been demonstrated. In addition, it has been shown that the host cells also participate in determining the morphology of the transformed cells and in determining whether or not a cell becomes infected by the virus. As previously discussed, the moderate nature of RSV leads one to suspect the existence of host effects on the genetic character of the virus. But the available evidence on this point is no more than suggestive.

These observations offer a mechanism, not only for RSV-induced tumors, but possibly more generally, to explain variation in virus-induced tumors. However, these observations do not explain why RSV should cause tumors. The tumor-inducing ability of the virus has been constantly associated with it for the 50 years of its study. The present work establishes that RSV has some kind of a close relationship with the genome of the infected cell. It is reasonable to assume that this relationship causes the cell to become malignant. From a general standpoint, there are two possible means for this to happen; the virus could contribute information to the cell necessary for the cell to become malignant; or the virus could activate a pre-existing potential malignant state of the cell. The conversion of the same type of cell to different types of Rous sarcoma cell by mutants of the virus indicates that the virus can contribute information which determines the character of the Rous sarcoma cell. However, whether this occurs because the virus contributes information for malignancy, or because the differentiating action is independent of the carcinogenic action is not known.

The only physiological difference yet found between Rous sarcoma cells and normal cells in vitro is the ability of the Rous sarcoma cells to grow under conditions preventing the growth of the normal cells, e.g., extreme crowding. The mechanism for this difference is not yet known.

Relevance for comparison of RSV and other animal viruses. With the aid of the techniques developed in the course of this work, it has been possible to study RSV in a way comparable to that used for cytocidal animal viruses. In spite of the moderate nature of RSV, this virus shows a number of similarities to other animal viruses. The characteristics of its entrance into cells, adsorption and penetration, appear not different from those of other animal viruses. The study of viral growth curves does not reveal anything strikingly characteristic for RSV. In fact, if the curves reported here are compared with those of Western equine encephalomyelitis virus (Dulbecco and Vogt, 1954), of Newcastle disease virus (Levine and Sagik, 1956), and of vesicular stomatitis virus (VS) (Franklin, 1958) on the same cell, one finds a considerable qualitative similarity; only some quantitative differences can be found. Of these, perhaps the most significant is the length of the latent period, which is three to four times longer for RSV than for the other viruses. The longer period may be indicative of a more complex initial interaction between RSV and the cell, an idea also supported by the radiobiological findings. Another difference is the yield of infectious virus from Rous sarcoma cells. Rous sarcoma cells release at most 40 FFU during every 20 hour period. Cells infected with the other animal viruses release relatively much larger amounts of virus in a shorter period of time.

The radiobiological experiments do reveal a striking difference between RSV and other animal viruses in the sensitivity of the capacity of the uninfected cell to support virus growth. However, the evolution of this sensitivity after infection again shows similarities between RSV, polio virus (Dulbecco and Vogt, 1955; Dulbecco, 1956) and VS (Franklin, 1958). In fact, in all cases the Luria-Latarjet curves tend to decrease in sensitivity -- although to a very different extent -- and are of approximately single hit type. The data for RSV would thus support Dulbecco's hypothesis (1956) of one or two centers for virus reproduction in animal cells. The observations on the single particle requirement for interference with RSV may also support this idea.

Apparently differences exist between the nature of the centers involved in the integration of RSV and of the other viruses. On the basis of identical radio-sensitivity, the center responsible for the integration of RSV appears to be responsible also for the multiplication of the uninfected cells. In polio and VS, on the contrary, the center appears to be formed essentially by the genome of the infecting virus, since the initial capacity has a radiosensitivity corresponding to that of the free virus. The final virus-cell complex of RSV also appears to be formed essentially by the genome of the infecting virus on the basis of similar radiosensitivity.

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APPENDIX

Photos 1 - 4	From	plate 1	Temin and Rubin, 1958 Virology 6, 574
Fig. 1	"	Fig. 1 of	Temin and Rubin, 1958 Virology 6, 675
Fig. 2	"	Fig. 4 of	Temin and Rubin, 1958 Virology 6, 680
Fig. 3	"	Fig. 8 of	Temin and Rubin, 1958 Virology 6, 681
Fig. 6	"	Fig. 1 of	Temin and Rubin, 1959 Virology 8, 213
Fig. 7	"	Fig. 2 of	Temin and Rubin, 1959 Virology 8, 218
Photos 5 - 7	"	plates of	Temin, 1960 Virology 10, 182

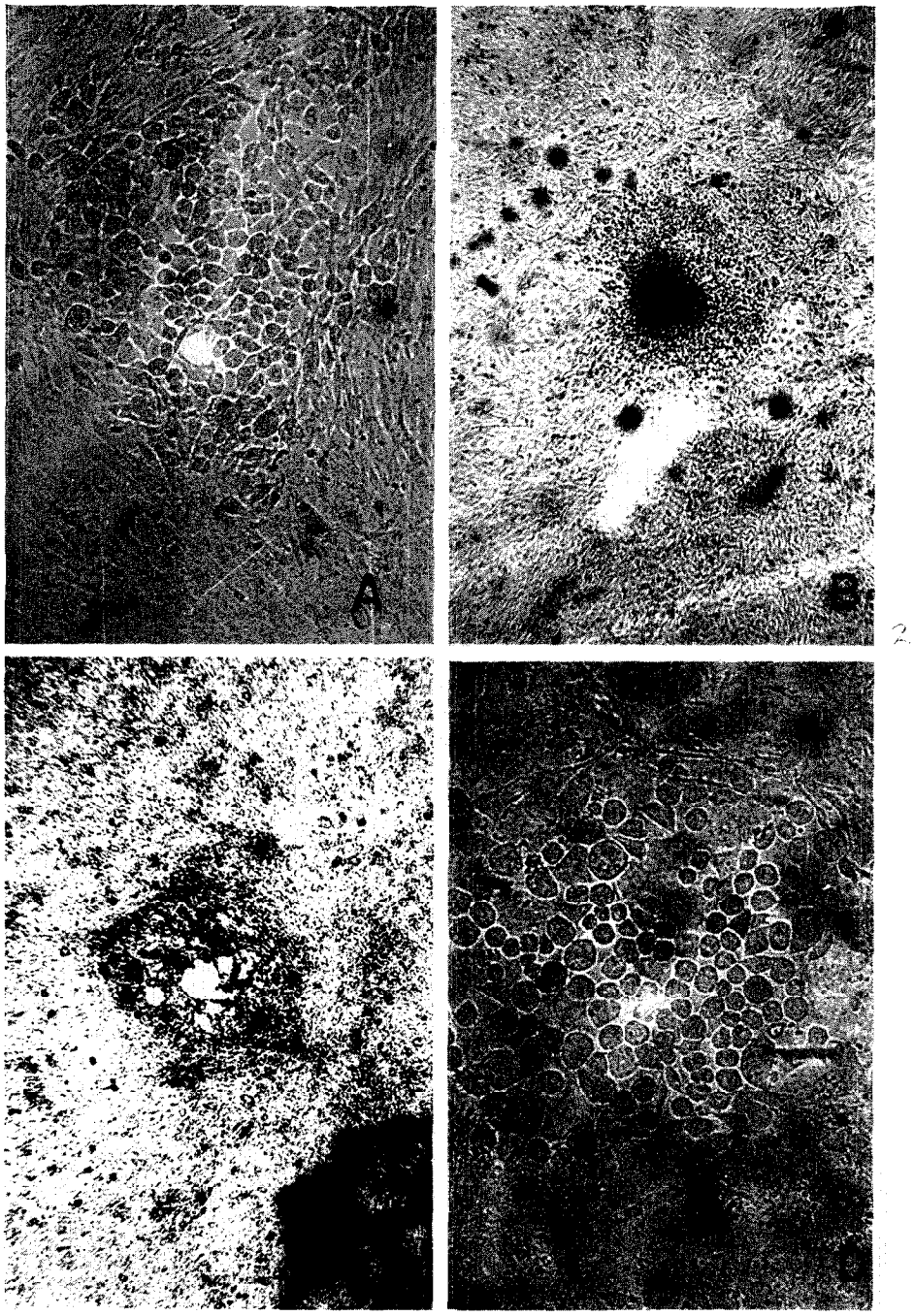
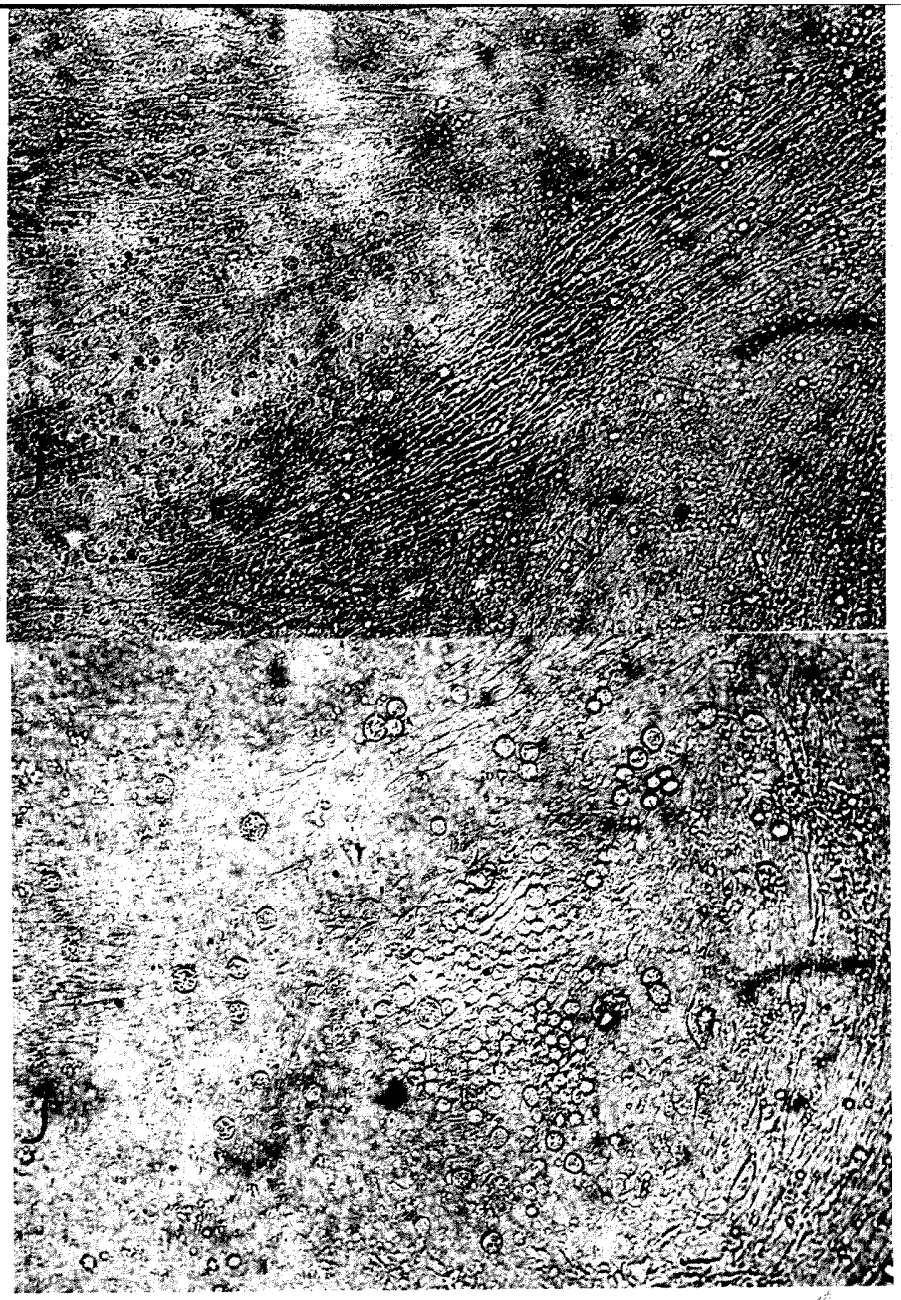
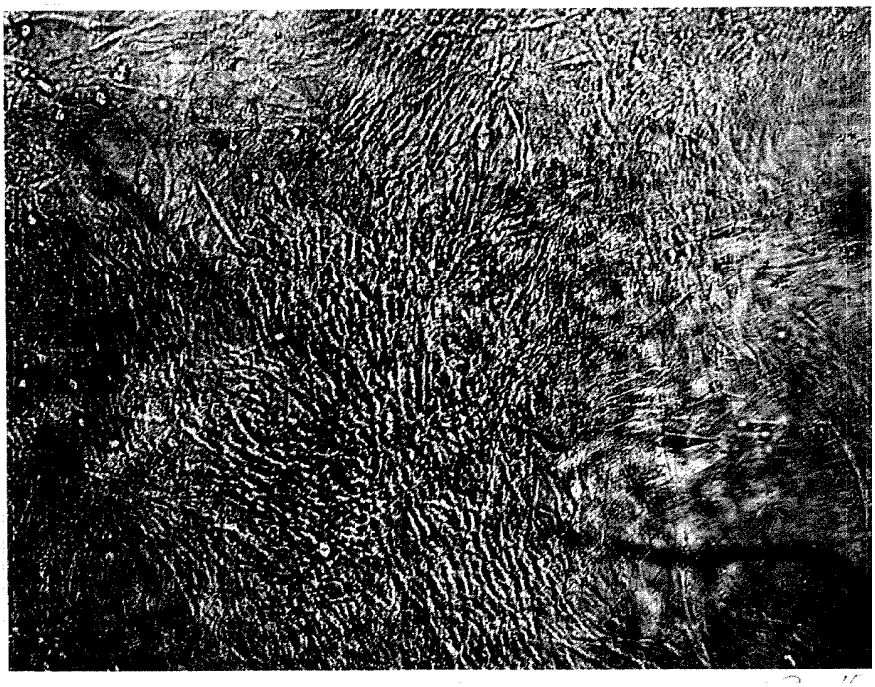


PLATE I





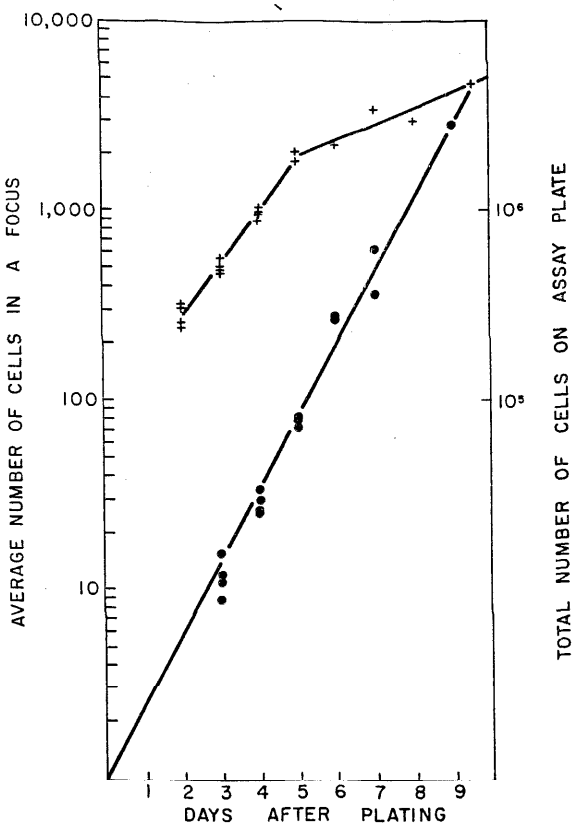


FIG. 1. Increase in number of cells in a focus. Rous cells were plated on assay plates. Twenty hours later agar was added to the plates. Every day the number of cells in 10–20 foci was counted. The total number of cells on the plate was also counted: first, by directly counting a fraction of the cells on the plate; and after the third day, by removing the agar, trypsinizing the cells, and counting in a hemocytometer. ● = Average numer of cells in a focus. + = Number of cells on an assay plate.

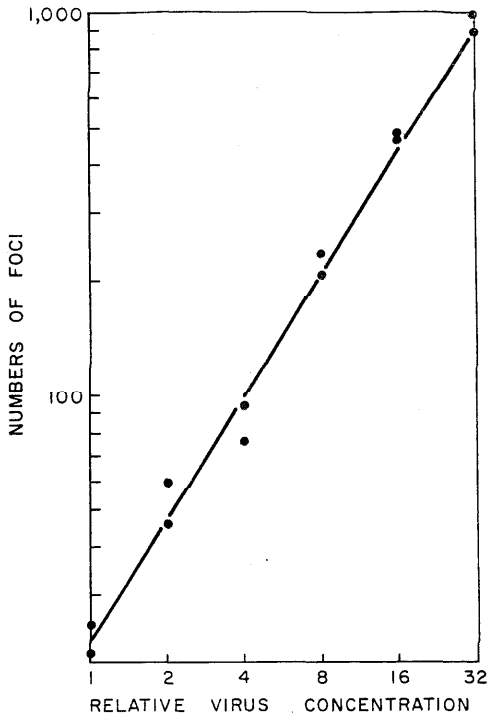


FIG. 4. Relationship between virus concentration and number of foci. A series of twofold virus dilutions between 1/80 and 1/2560 of a virus stock containing 7×10^4 FFU/ml were placed on secondary cultures for 30 minutes and agar added. To enable counting of as many as 1000 foci per plate the cultures were counted on the fifth day after infection.

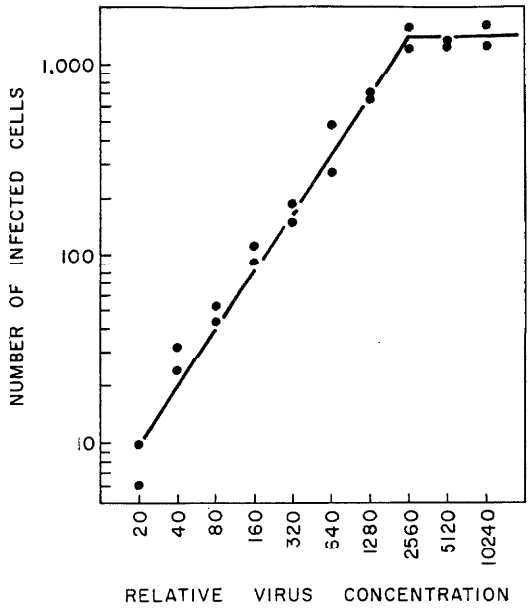


FIG. 3. Relationship between virus concentration and number of infected cells. Virus dilutions from undiluted to 1/512 of a stock containing 10^5 FFU/ml were placed on secondary cultures for 30 minutes. The cultures were washed, trypsinized, and various dilutions of the infected cell suspension were placed on each of two secondary cultures. Sixteen hours later agar was added. The plates were counted on the sixth day after infection.

FIG. 4

Kinetics of adsorption. An inoculum of virus containing about 50 FFU in 0.2 ml was added to each of several secondary cultures. After adsorption for varying lengths of times the supernatant was removed and an agar overlay added. Two separate experiments are shown. Each point is the average of two assay plates.

FIG. 4

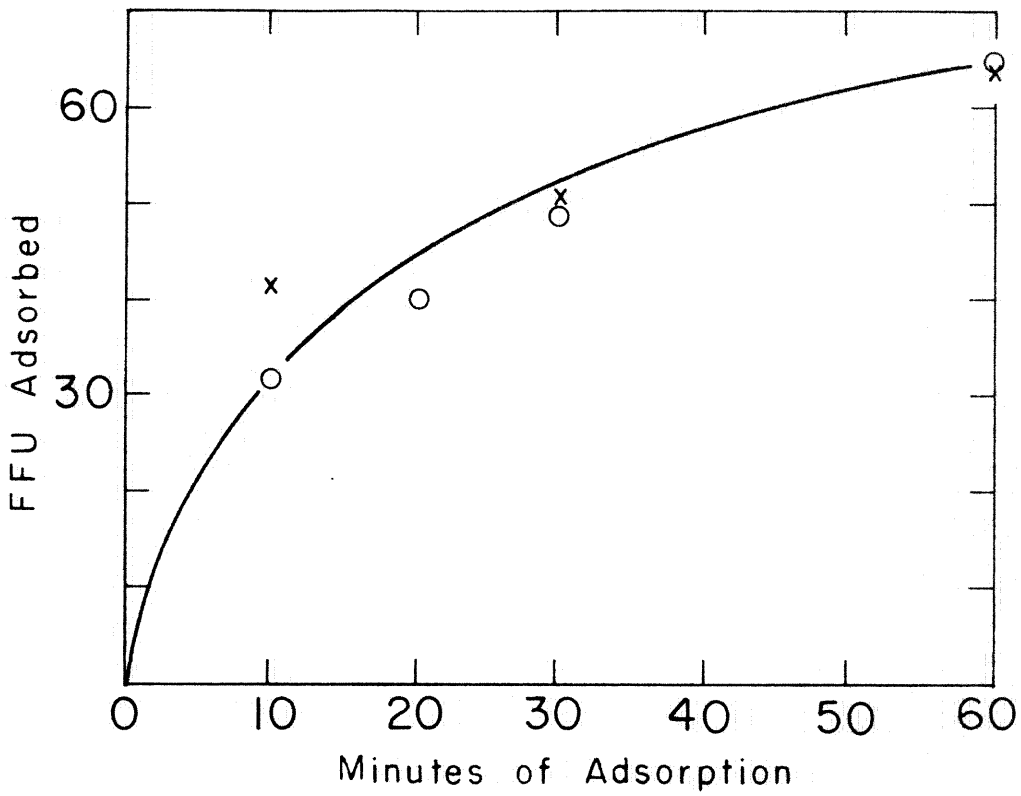
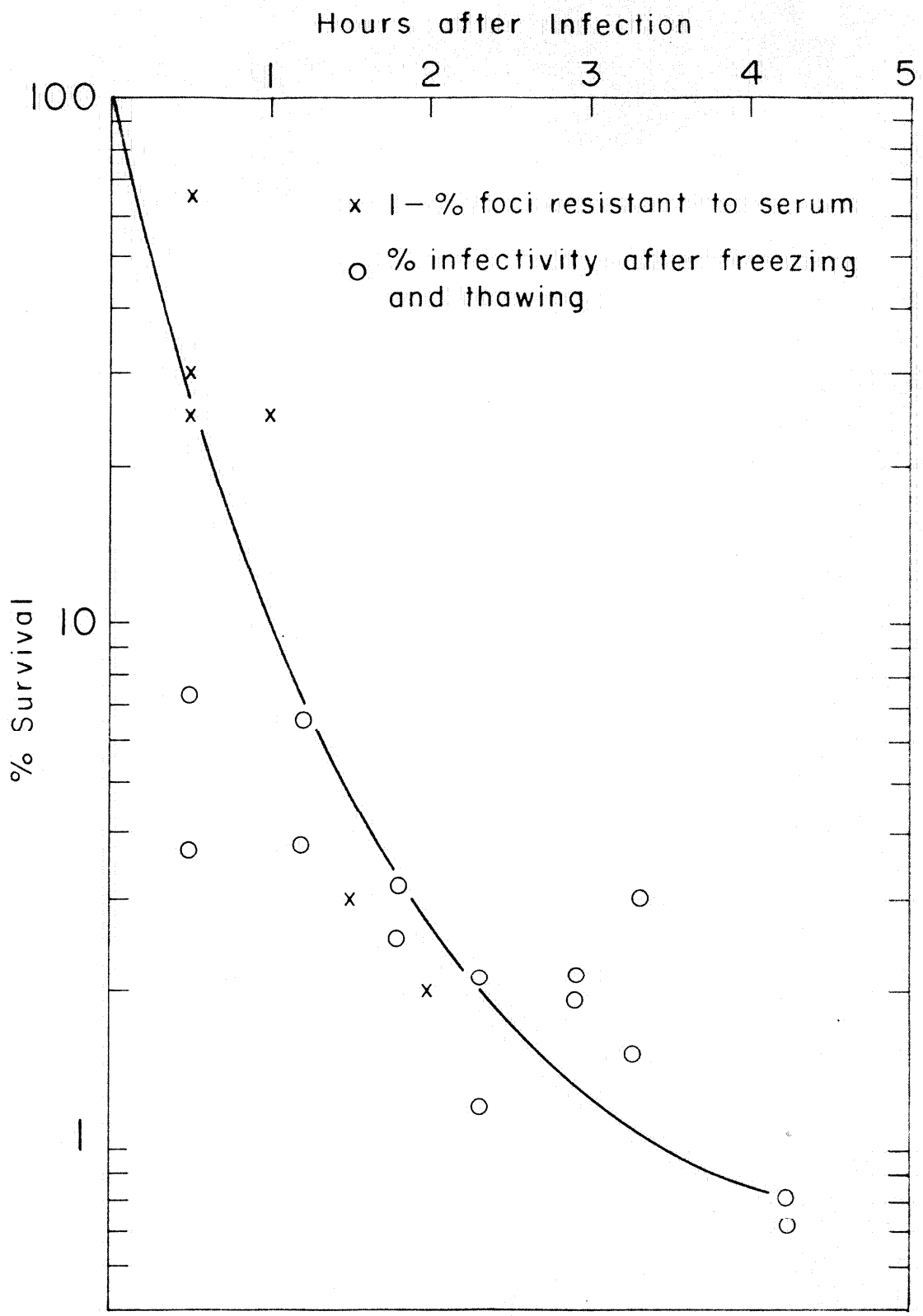


FIG. 5

Kinetics of penetration. Several secondary cultures were infected with virus for 30 minutes, the supernatants removed, and the plates washed twice with medium. Three ml of medium was added. At intervals thereafter neutralizing serum was added to one culture for half an hour. A parallel culture was trypsinized, the cells disrupted by freezing and thawing three times and assayed for RSV. The results are plotted as per cent foci surviving serum as compared to an untreated control, and per cent RSV surviving compared to a culture disrupted immediately after infection.

FIG. 5


Hours after Infection
1
2
3
4
5
100
10
1
% Survival
x 1 – % foci resistant to serum
o % infectivity after freezing and thawing

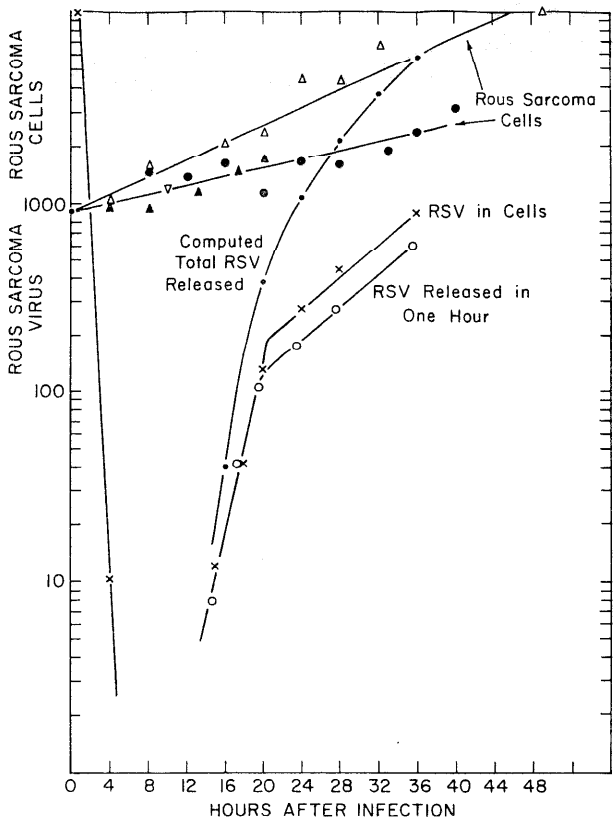


FIG. 5. Growth curves of Rous sarcoma cells and Rous sarcoma virus after infection. A number of secondary cultures of chick embryo cells were infected with 0.2 ml of an undiluted RSV stock containing 5×10^5 FFU/ml. After adsorption for 30 minutes, the unadsorbed virus was removed. The cultures were washed twice with 5 ml of Eagle's medium, and 5 ml of growth medium was added. Every 4 hours the supernatant was removed from one or two cultures and 1 ml of growth medium added. After incubation for 1 hour the supernatant was removed and assayed for RSV. The cells were placed in suspension with trypsin, counted, and 10^4 added to other secondary cultures to determine the number of Rous sarcoma cells. The remaining cells were placed in 1 ml of growth medium, disrupted by freezing and thawing three times, and assayed for cell-associated RSV. The curves for growth of Rous sarcoma cells are taken from several experiments. The growth rate appeared to be somewhat different from one experiment to another (upper two curves). The curve for rate of release of RSV has been integrated to give the total virus released. This curve cannot be evaluated directly because of the high rate of thermal inactivation of RSV at 37°. Each point is the average of two or more plates. △, ▲, ▽, ● = Growth of Rous sarcoma cells in different experiments; × = cell-associated RSV; ○ = RSV released in 1 hour; • = total RSV released (integral of RSV released in 1 hour).

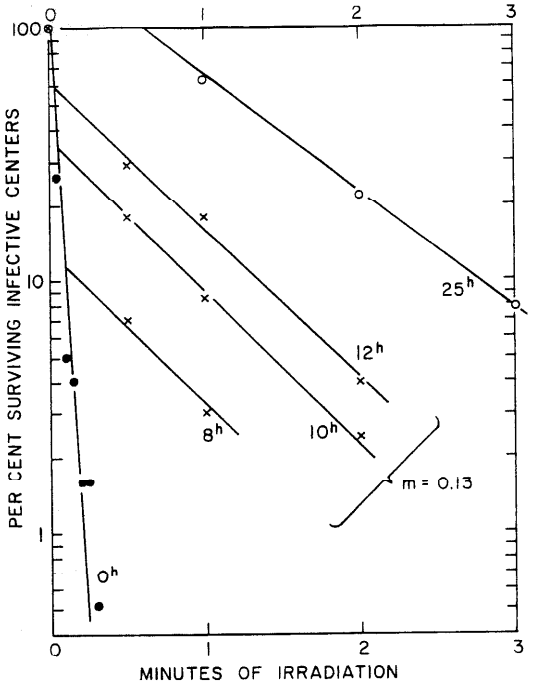


FIG. 7. Shift in radioresistance of capacity of cells to release RSV after infection. Several secondary chicken embryo cultures were infected with 0.2 ml of an RSV stock containing between 5×10^4 and 5×10^5 FFU/ml. After adsorption for 30 minutes the unadsorbed virus was removed and 3 ml of growth medium added. At 0, 8, 10, 12, and 25 hours after infection the cells were placed in suspension with trypsin, counted, irradiated with X-rays and aliquots plated for infective centers. The curves are taken from three separate experiments. Each point is the average of two plates.

FIG. 8

Number of particles needed for interference. Stocks of virus were inactivated by incubation at 37° or by irradiation with ultra-violet light. Dilutions of inactive virus were placed on assay plates for 30 minutes, supernatants removed and 100 FFU of RSV added. The number of foci are plotted as per cent of the medium treated control.

FIG. 8