



A comparative study of the Schmidt-Ruppin and Bryan high titer strains of Rous sarcoma virus
by Clayton Arthur Buck

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Bacteriology

Montana State University

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Abstract:

The biological properties of the Bryan high titer strain of Rous sarcoma virus, RSV(B) , and the Schmidt-Ruppin strain, RSV(SR) , were compared. RSV(B) was found to be more pathogenic, in vivo and in-vitro than RSV(SR). RSV(B) carried neutralizable antigens not found on RSV(SR) and was 3 times more heat stable than RSV(SR). Defectiveness was found to be a property of RSV(B) but not of RSV(SR). Both RSV(SR) and RSV(B) were produced at the same rate by infected cells; however, there was always 10 to 100 times more RSV(B) produced than RSV(SR). Puromycin had identical effects on 24 hr. RSV(B) and RSV(SR) production; however, the effects of puromycin on 48 hr. RSV(SR) production appeared to be different from its effects on 48 hr. RSV(B) production. Both viruses were inhibited by the addition of actinomycin D to cells within the first 35 hrs. after infection. Possible explanations of the differences between the 2 viruses were discussed.

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STRAINS OF ROUS SARCOMA VIRUS

by

CLAYTON ARTHUR BUCK

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of

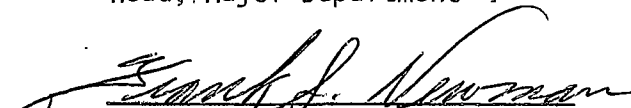
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ABSTRACT

The biological properties of the Bryan high titer strain of Rous sarcoma virus, RSV(B), and the Schmidt-Ruppin strain, RSV(SR), were compared. RSV(B) was found to be more pathogenic, in vivo and in vitro than RSV(SR). RSV(B) carried neutralizable antigens not found on RSV(SR) and was 3 times more heat stable than RSV(SR). Defectiveness was found to be a property of RSV(B) but not of RSV(SR). Both RSV(SR) and RSV(B) were produced at the same rate by infected cells; however, there was always 10 to 100 times more RSV(B) produced than RSV(SR). Puromycin had identical effects on 24 hr. RSV(B) and RSV(SR) production; however, the effects of puromycin on 48 hr. RSV(SR) production appeared to be different from its effects on 48 hr. RSV(B) production. Both viruses were inhibited by the addition of Actinomycin D to cells within the first 35 hrs. after infection. Possible explanations of the differences between the 2 viruses were discussed.

INTRODUCTION TO THE VIRUS

Rous and His Virus

The tumor which was to eventually give rise to virus was reported by Rous in 1910. He designated it Chicken Tumor 1. It arose on the breast of a 15 month old Plymouth Rock hen and was removed 2 months later. Bits of the tumor were inoculated into the original hen and into 2 other hens of the same stock. Originally the tumor grew very slowly and only in Plymouth Rock birds. In birds other than his laboratory stock, Rous obtained transient growth which eventually regressed. During the first few transfers of the tumor, the growths which resulted were encapsulated and only rarely gave rise to metastases. No attempts were made to isolate virus from the original tumor. Attempts to grow tumor transplant in pigeons and guinea pigs were unsuccessful.

A year later 2 reports appeared (Rous, 1911a; 1911b) in which Rous had successfully induced sarcomas in chickens using a filterable agent which was later to be referred to as Rous sarcoma virus. The filtrate was prepared (Rous, 1911a) by grinding 15 gm of transplanted Chicken Tumor 1 in a warm mortar with sand and adding Ringer's salt solution. The debris was removed by centrifugation and the supernate filtered through a number 5 Berkefeld filter. The entire operation was performed at 38.5 C, the body temperature of a chicken.

By this time, the tumor had shown a definite increase in virulence (Rous, 1911b). Tumors now appeared within 7 days of inoculation instead of the original 30 to 35 days. The tumors always gave rise to metastatic nodules, whereas, up to this time, metastases were noted only once (Rous,

1910). Finally, tumors could be induced in chickens of other strains (Rous, 1911b). However, attempts to induce tumors in rats, guinea pigs, rabbits, ducks, and pigeons still failed (Rous, 1911b).

In 1913, Rous and Murphy discussed some pathological changes that the tumor had undergone. Originally (Rous, 1910), the tumor was an encapsulated spindle cell sarcoma which grew rather slowly. By 1913, the tumor, whether induced by cells or a cell-free filtrate, was a soft, friable, non-encapsulated sarcoma which grew and metastasized rapidly. The typical cells varied from spindle cells to short, blunt, or rounded cells, with giant cells frequently being found. The tumor was hemorrhagic and produced large quantities of a mucinous material. This 1913 form of the tumor resembled the tumors which were obtained in this laboratory when Rous sarcoma virus was injected into the wing web of chickens (Materials and Methods).

Rous continued working with his filterable agent and characterized it in a manner which was not considerably improved upon until extensive in vitro work with the virus was made possible (Temin and Rubin, 1958). The most extensive general physico-chemical characterization of the virus was published by Rous and Murphy in 1912. They found that the virus could be stored in dried tissue for up to 7 months or in 50 per cent glycerin for 1 month. The agent, as a ground tumor suspension, could survive rapid freezing and thawing and also heating to 53°C for 15 min, but it could not survive heating to 55°C for 15 min. These results correlate closely with those of Dougherty (1961). Rous and Murphy (1912) also demonstrated the sensitivity of the agent to surface active materials such as bile and

Saponin as well as chloroform, toluol and alcohol; thereby indicating, as Friesen and Rubin (1961) have, that lipids probably play an important role in the activity of the virus.

In 1911, Rous and Murphy reported that discrete tumors could be induced on the membranes of developing chick embryos with a filtrate from Chicken Tumor 1. It is unfortunate that they did not continue this work past the pathological characterization of the tumors as they were working, evidently unknowingly, with the first good means of quantitating this virus. This aspect of Rous sarcoma virus work had to wait 27 years (Keogh, 1938) for development. Rous and Murphy (1911) insinuated that they might be able to induce tumors in utero in mice and rats with their tumor extracts. No further positive reports seem to have been published concerning this work, but it may well have been a prelude to the origin of strains of Rous sarcoma virus whose host ranges extended beyond that of the chicken (Duran-Reynolds, 1947; Zilber and Kryukova, 1957; Munroe and Windle, 1963; Ahlström et al., 1962).

Rous (1913) was able to show that birds responded separately to viral and tumor antigens. He found that birds which had managed to reject their tumors could not be infected with either tumor cells or filtrates. He also was able to obtain birds which were resistant to filtrate challenge, but would develop tumors if whole cells were implanted in their breasts. He, therefore, concluded that the virus and cells were able to act either separately or together to induce tumors. This work was confirmed and extended nearly 50 years later by Hanafusa et al. (1964), Dougherty and Morgan (1962), and Temin (1962).

Rous et al. (1919) attempted to produce neutralizing antibodies against the filterable agent by injecting ground tumor extracts as well as tumor cells into rabbits. They succeeded in obtaining only good anti-chicken serum. They finally obtained neutralizing antibodies by injecting geese intravenously with tumor suspensions and blood. The sera obtained would stop tumor production by the filtrate if the serum and filtrate were mixed prior to challenging the chickens. After adsorption with chicken tissue, the goose-anti-Chicken Tumor 1 serum would still neutralize the infecting agent. Rous and his colleagues concluded that anti-chicken sera would not neutralize their infective agent. It is interesting that Gye and Purdy (1933) reported neutralization of the Rous agent with anti-chicken serum. This report has been successfully repudiated by Rubin (1956; 1961) and by Fink (1964). Their evidence against Gye and Purdy's data (1933) was based upon the fact that anti-chicken sera could prevent tumor development by interacting with the infected tissue (Rubin, 1956), and the fact that virus neutralizing antibodies persisted even after extensive adsorption of rabbit anti-RSV sera with chicken tissue (Fink, 1964).

Rous and his filterable agent helped lay the foundation for a controversy which continues to plague avian leukosis research. While working with chicken tumors, Rous isolated 3 tumors, Chicken Tumors I, VII, and XVIII, which were transmissible by a filterable agent (Rous and Murphy, 1913b). The obvious question arose. Were these 3 different manifestations of the same agent or single manifestations of 3 related agents? The 3 agents were alike in that all could be stored within desiccated tumor tissue, all passed through some Berkefeld filters but were retained by

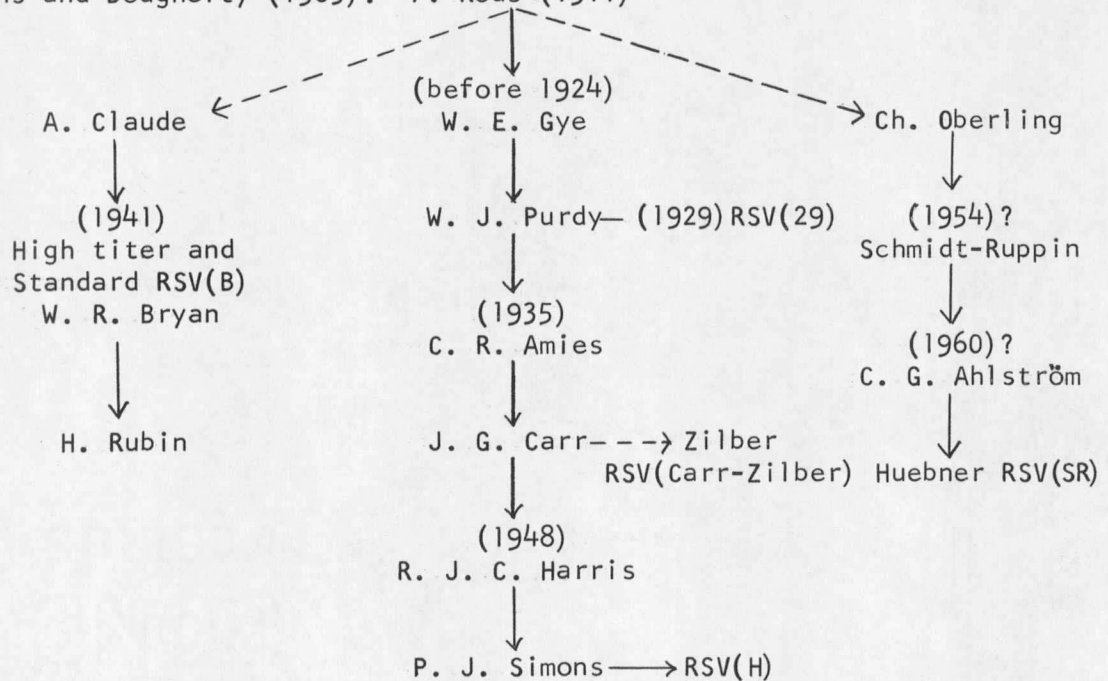
extremely fine ones, and all produced malignancies in chickens (Rous and Murphy, 1913b). Therefore, Rous and Murphy (1913b) suggested that they were all 3 of 1 class, "whatever that class may be". However, since Chicken Tumor I virus always gave rise to a spindle-celled sarcoma (Rous, 1911a) and Chicken Tumor VII virus, to an osteochondrosarcoma (Rous et al., 1912) and Chicken Tumor XVIII virus, to a spindle-celled sarcoma with a peculiar intraconalicular pattern (Rous and Lang, 1913), it was concluded that these 3 agents composed a new group of entities capable of causing neoplasms of diverse characters in chickens (Rous and Murphy, 1913b). However, Rous and Murphy pointed out the uncertainties of classifying the agents strictly on the basis of tumor pathology when they showed that, during the selection for rapidly growing Chicken Tumor XVIII virus, the histology of the tumor came to resemble more closely that of Chicken Tumor I. In 1914, however, they demonstrated that chickens which were resistant to Chicken Tumor VII were not resistant to Chicken Tumor I (Rous and Murphy, 1914); thereby, opening new paths for tumor virus classification and identification and supporting their contention that these were separate viruses.

Rous was not quick to conclude that he was dealing with a virus. Indeed, in discussing the filterable agent (Rous, 1911b), he states that the first tendency was to regard it as a self-perpetuating parasitic organism, but he also felt that it was possible that tumor cells secreted a chemical stimulant which resulted in neoplastic changes in affected cells causing them, in turn, to release the chemical. After his work on characterization of the agent (Rous and Murphy, 1912), he concluded that it was a

living organism and in 1913 (Rous and Murphy, 1913a), referred to the agent as a "causative virus". The fact that Peyton Rous, less than 15 years after Beijerinck's introduction of the concept of filterable viruses (Beijerinck, 1899), was able to isolate a virus, recognize it as such, characterize it physically, chemically and antigenically, and suggest that it was only 1 of a group of avian tumor viruses which might serve as a model for the cause of cancer in man, was a tribute to his scientific abilities and insight.

Genealogy of Rous Sarcoma Virus

The diversity of conditions and the selection for various characteristics in the many laboratories over the past 50 years have resulted in several "strains" of Rous sarcoma virus (RSV) with differing characteristics. The following genealogy of RSV is a modification of that published by Simons and Dougherty (1963): P. Rous (1911)



The dotted lines represent conjecture; the dates in parentheses represent approximate times of receipt of the virus stocks; the known strain designations are listed whenever possible following the name of the individual using the designation.

These various strains of RSV have their own distinguishing characteristics. RSV(B) carries high concentrations of Rous Associated Virus (RAV) (Rubin and Vogt, 1962) and forms sharp, distinctive foci in tissue culture (see below). RSV(SR) is not defective, produces foci which are quite distinct from those of RSV(B) and will not react with RSV(B) neutralizing sera (see below). RSV(H) is antigenically distinct from RSV(29), produces no foci in tissue cultures, and produces tumors on the chorio-allantoic membrane of chicks which are distinguishable from those of RSV(29) and RSV(B) (Dougherty et al., 1963). RSV (Carr-Zilber) will infect mammals, including primates (Munroe and Windle, 1963). RSV(SR) will also infect mammals and cause chromosomal breaks in human cells (Ahlström et al., 1962; Nichols et al., 1964). Recently, Rabotti et al. (1965) have succeeded in inducing tumors in the brains of hamsters with RSV(B).

Rous Sarcoma Virus Classification

RSV is a member of the avian leukosis group of viruses (Jungherr et al., 1941). This group includes the viruses of avian lymphomatosis, erythroblastosis, myeloblastosis, and osteopetrosis, as well as various

solid tumor viruses such as the Fujinami virus (Beard, 1957). All the viruses of this group so far examined are helical RNA viruses (Howatson, 1964) which multiply in the cytoplasm of infected cells and are released at the cell membrane (Haguenau and Beard, 1962; Vogt and Rubin, 1961; Beard, 1963). Structurally, they resemble the myxoviruses in that they have an outer envelope enclosing an inner membrane which encloses the nucleoid (Howatson, 1964; Beard, 1963; Dourmashkin and Simons, 1961). This group of viruses shares a common soluble antigen which is found in virus particles and in neoplastic cells induced by the virus (Huebner et al., 1964). These viruses also share a number of neutralizing antigens, all of which are not reciprocally susceptible to the same antibodies (Beard, 1957; Simons and Dougherty, 1963; Friesen and Rubin, 1961).

Assay of Rous Sarcoma Virus

Probably the most valuable contribution to the study of RSV has been the development of an in vitro assay system (Temin and Rubin, 1958). This system relies on the formation of foci of transformed cells on a monolayer of chick fibroblasts. The number of foci on a petri dish was shown to be directly proportional to the amount of infecting virus until 1 to 10 per cent of the cells were infected (Temin and Rubin, 1958); therefore, if the number of foci on a plate were kept within the limits of statistical accuracy, the titer of a virus stock could be expressed in focus forming units (FFU) per ml of virus.

Direct microscopic comparison of the number of viruses in a culture and the number of foci obtained by focus assay showed that there were about

750 virus particles present for each focus produced (Crawford and Crawford, 1961). However, they calculated that there might actually be 15 particles per FFU if one assumed that the use of non-confluent monolayers lowered the efficiency of the assay system and that only 10% of the cells were infected. Considering the rapid loss of viability of viruses due to heat inactivation (Rubin, 1955), the system would seem to be rather efficient.

This system has been plagued by many difficulties. First, focus formation may be suppressed by having too many cells on the plate at the time of infection (Rubin, 1960b). Second, the use of fetal calf serum, Fetuin, or over 10% calf serum suppresses focus formation (Rubin, 1960c). Third, Prince (1962) found that the omission of tryptose phosphate broth (Difco) resulted in the suppression of focus formation.

Besides the influence of factors in the medium, Rubin (1960a) found that the embryos from certain eggs carried a virus, Rous interfering factor (RIF), which interfered with RSV infection and thus stopped focus formation. This discovery led to the use of interference assay for avian leukosis viruses (Rubin, 1960a; Rubin and Vogt, 1962), which in turn led to the discovery of the interfering virus carried in RSV stocks, Rous associated virus (RAV). The fact that RAV was found to be present in concentrations up to 10 times that of RSV (Hanafusa et al., 1963) explained why only 1 to 10 per cent of the cells in a plate could be infected with RSV. Besides obtaining a reduction of foci due to viral interference, Rubin (1964) and Crittenden et al. (1963) have shown that certain strains of chickens give rise to genetically resistant embryos which cannot be infected with RSV.

Defectiveness of Rous Sarcoma Virus

In 1962, Rubin and Vogt reported finding 2 viruses in their stocks of the Bryan high titer strain of RSV. One was RSV itself and the other they called Rous associated virus (RAV). RAV was always present in much higher concentrations than RSV. Subsequently, Hanafusa et al. (1963) reported that foci of RSV transformed cells which were produced on plates infected with high dilutions of RSV would not produce virus. They also showed that these non-producing (NP) cells would produce RSV only if superinfected with RAV or some other avian leukosis virus (Hanafusa et al., 1963; 1964a). Thus, it was shown that RSV depended upon a helper virus for maturation. They concluded that the RSV genome lacked the information necessary for maturation within an infected cell and RSV must, therefore, be defective. A word of caution should be injected here. Although Hanafusa et al. (1963; 1964a) report that RAV was found in all RSV cultures tested, there was no data to indicate that RSV(H), RSV (Carr-Zilber), or RSV(29) were tested. One should not conclude that all strains of RSV are defective since only RSV(B) has been extensively studied, and, as will be shown below, RSV(SR) has been found not to be defective.

Properties of RSV Conferred by Helper Viruses

Hanafusa et al. (1964a) were able to show that the RSV, produced by NP cells as a result of induction, carried the neutralizing antigen of the helper virus. That is, if the NP cells were induced by an avian lymphomatosis virus, the resulting RSV could be neutralized by specific anti-avian lymphomatosis sera. Therefore, upon infecting a cell, RSV was shown to

cast off its protein coat and exchange it for that of any helper virus which might be present. The helper that was most handy was, of course, RAV, but by proper manipulation, any avian leukosis virus could serve as the helper and thus contribute the coat of the resulting RSV particles. This was very much like phenotypic mixing (Novick and Szilard, 1951; Streisinger, 1956) except that only 1 of the viruses, i.e., RAV, contributed the coat protein.

Hanafusa et al. (1964b) showed that induced NP cells produced both RSV and RAV at the same time, and that the rate of virus release was similar to that of RAV alone. Later, Rubin concluded (1964) that the rate limiting step in RSV production was the coating of the virus. This meant that the rate at which virus was released from the cell was determined by the helper virus.

A second property of RSV which has been shown to be affected by the helper involves viral interference. Rubin (1960a) found that infection of chick fibroblasts with an avian leukosis virus prior to exposure of the same cells to RSV would prevent RSV infection. Interference was shown also to be a property induced by RAV (Rubin and Vogt, 1962). Hanafusa (1965) and Vogt (1965) isolated 2 strains of RAV. They were designated RAV 1 and RAV 2. The 2 RAV's were found to be antigenically distinct and differed in their ability to infect certain strains of chick fibroblasts. For convenience, the RSV genome that was inside a RAV 1 coat was designated RSV(RAV 1), and that which was in a RAV 2 coat, RSV(RAV 2). If chick fibroblasts were infected with RAV 1, and superinfected with RSV(RAV 1), no focus formation

occurred. If, however, the RAV 1 infected cells were challenged with RSV (RAV 2), focus formation, and therefore infection, occurred. Thus, susceptibility to interference was found to be a property of the virus coats and hence a property governed by the helper virus. Interference would seem to be, therefore, a property involving virus adsorption and penetration into a cell rather than a physiological competition between 2 viruses. Indeed, the RSV(B) producing cell supports the growth of 2 viruses which must be physiologically compatible.

RAV was also found to influence the ability of RSV to infect certain chick fibroblasts (Vogt, 1965; Hanafusa, 1965). The fibroblasts which were resistant to RAV 2 were also resistant to RSV(RAV 2), but not to RSV(RAV 1).

Even though properties involving functions of the virus coat have been shown to be determined by the helper virus, the outstanding property of RSV, its ability to transform normal cells into tumor cells, has been shown to be exclusively a property of the RSV genome (Hanafusa et al., 1964a; Rubin, 1964; and Temin, 1962). Thus, non-producer cells induced in vitro could cause tumor formation if injected into the wing webs of chickens (Hanafusa et al., 1964a), and these tumors still could not produce virus unless they were infected with RAV. This might explain the rather common occurrence of non-virus yielding tumors (Shimizu and Rubin, 1964). The infection of birds with low doses of RSV or the infection of birds having anti-leukosis antibodies could result in such non-producing tumors by preventing RAV infection of RSV cells. However, it should be kept in mind that material has been found in tumor extracts which will neutralize RSV (Andrews, 1932;

Dougherty et al., 1960). This could also explain why RSV might not be obtained from some tumors.

The Effects of Inhibitors on Rous Sarcoma Virus Replication

Since ribonucleic acid (RNA) was shown to constitute the genetic material of RSV (Bather, 1957; Epstein and Holt, 1958; Crawford and Crawford, 1961), it was only logical that attempts would be made to inhibit RSV replication using antimetabolites which were known to affect RNA synthesis.

Goldé and Vigier published work showing that RSV replication could be inhibited by 5-fluoruracil (FU) (Goldé and Vigier, 1961 and 1963). They found that the addition of 20 $\mu\text{g}/\text{ml}$ of FU to virus infected cells 1 to 10 hrs. after infection inhibited virus production at 24 hrs. They also found that the addition of the same concentrations of FU between 1 and 20 hrs. after infection suppressed 48 hr. virus production. They discovered, however, that cells infected with RSV for 3 days became completely refractile to the effects of FU. The activity of FU could be reversed by the addition of uracil (200 $\mu\text{g}/\text{ml}$) to the medium. Thymidine would not reverse these effects. On the basis of these experiments, they concluded that there was at least 1, and possibly 2, FU sensitive steps involved in RSV infection. The first occurred between 1 and 10 hrs. after infection and was necessary for virus production at 24 hrs. The second occurred between 5 and 20 hrs. and was necessary for virus production at 48 hrs. It is possible that up to 3 days were required for all their infected cells to begin producing

virus and that later additions of FU were merely blocking the same early steps in the cells which were just beginning to synthesize virus.

Following this, work was published on the use of actinomycin D (AD), amethopterin (AM), and mitomycin C (MC) to stop viral replication. Temin (1963) showed that AD at concentrations from 0.1 to 10 $\mu\text{g}/\text{ml}$ would stop RSV multiplication. He found that the addition of 0.2 $\mu\text{g}/\text{ml}$ of AD to cells from 8 hrs. before to 24 hrs. after infection irreversibly stopped RSV production. If, however, 0.1 $\mu\text{g}/\text{ml}$ of the antibiotic was added to cells that had been carrying RSV for several generations (established Rous cells), and removed 8 hrs. later, the cells recovered their ability to produce virus within 12 to 16 hrs. after treatment. Thus, AD would reversibly inhibit virus production in established Rous cells. Temin confirmed and extended these results with experiments using AM (Temin, 1964a). He found that 10^{-7} M AM had no effect on virus production by established Rous cells, but like AD, it would inhibit virus production if it was added soon after infection, i.e., 1 to 4 hrs. The early inhibition of virus production by AM was, however, reversible. This was in contrast to the early effect of AD which was irreversible. Since AD acts by binding to deoxyribonucleic acid (DNA) molecules, thus preventing messenger RNA synthesis (Goldberg *et al.*, 1962), Temin concluded (1963; 1964a; 1964b) that RSV existed and functioned within the cells in a provirus state, and that the provirus was composed of DNA. Accordingly, upon infection, the RSV genome which was RNA, entered the cell and acted as a template for the synthesis of its DNA provirus. The provirus then directed virus production by the cell. Temin added strength to

his hypothesis by demonstrating that there was a larger amount of DNA in virus infected cells that would anneal with viral RNA than there was in non-infected cells (Temin, 1964b). Although Temin showed a consistent difference in the amounts of viral RNA which would anneal with DNA from infected and non-infected cells, the difference was not great. Also, in view of the fact that RSV(B), which Temin used, is defective, the hybridization demonstrated must have been between RAV RNA and DNA, not RSV RNA and DNA. In view of these facts, the work will need confirmation, preferably with a non-defective strain of RSV, using more sensitive techniques.

Vigier and Goldé (1964) obtained the same results with AD as did Temin (1963). They also used MC and found that, as with AD, the addition of MC within 24 hrs. after infection resulted in irreversible suppression of virus production. However, unlike AD, MC would not significantly inhibit virus production if added to established Rous cells at concentrations that did not produce cell death.

On the basis of their data, Vigier and Goldé proposed four models to explain the early sensitivity of the virus to these antimetabolites. First, an early enzyme coded by host DNA could be required to uncoat the viral RNA. This model would find precedence in the discovery of such an enzyme for uncoating pox viruses (Abel, 1963). Second, a special enzyme coded by host DNA might be required to open a secondary structure of RSV RNA. This model was based upon the inhibition of replication of reovirus 3, which is a double stranded RNA virus (Gomatos and Tamm, 1963), by AD and MC (Gomatos et al., 1962); however, Temin (1964b) showed that RAV RNA and RSV RNA were

sensitive to ribonuclease indicating that they are probably single stranded RNA's. Third, the RNA dependent RNA polymerase necessary for viral RNA synthesis might be coded by cellular DNA, or fourth, Temin's provirus model might be used to explain the results.

Of these 4 hypotheses, the first seems most likely since such an uncoating enzyme has been isolated. The possibility exists for a double stranded RNA to be found within the cell, but to date, AD has not been successfully combined with double stranded RNA (Goldberg and Reich, 1964). Therefore, direct interaction of AD with such RNA would not seem a likely explanation for viral inhibition. There has been no evidence presented that noninfected cells contain an RNA dependent RNA polymerase. In fact, its presence has been shown to be completely dependent upon the infecting virus (Baltimore et al., 1963). It is, therefore, difficult to understand why the cell would carry the information to code a molecule that it would never use as would be required by the third model. However, Weiss (1963) has reported that DNA dependent RNA polymerase can transcribe information in the form of polyribonucleotides directly off of RNA molecules. Therefore, it is possible that RSV RNA could compete with cellular DNA for the use of RNA polymerase and thereby require a cellular enzyme for replication. Temin's hypothesis and experiments have been based on the belief that AD, MC, and AM interference with RSV replication is unique since RSV is an RNA virus. However, AD has been found to interfere with influenza virus replication early in the multiplication cycle (Barry, et al., 1962; White et al., 1965). Influenza and RSV obviously do not behave the same within the cells.

Therefore, Temin's provirus postulation paralleling lysogeny is justified, and if his work can be confirmed in another laboratory, there would be little doubt that his DNA provirus state existed and must explain these data. But until that time, the first, or for that matter any of the other models, cannot be completely excluded.

Under any circumstances, the irreversibility of the first AD sensitive step is difficult to explain. If the uncoating enzyme model were adopted, one would have to assume that the virus, being rather unstable, loses its activity within the cell due to inactivation of viral nucleic acid at temperatures between 37 and 45°C. (Dougherty, 1961). If Temin's DNA provirus model were accepted, one must assume either that the nucleases of the cell break down the non-functioning DNA, which would be contradictory to reversible inhibition occurring in established Rous cells, or that at one time AD-DNA binding is stronger than at another. Also, in this case, one must explain why MC would attack the provirus DNA at one time and not at another. The MC data could be explained by the involvement of different sites on the host genome in early and late virus synthesis if one favors a hypothesis involving the host genome.

The later AD reversible (MC, AM, and FU insensitive) step is even more difficult to explain, as the sole adoption of Temin's idea has the built in contradiction stated previously. However, if one includes in Temin's hypothesis the necessity for cellular DNA integrity for virus release, a fairly reasonable explanation of both the early and late AD sensitive steps is possible.

One very important fact has been omitted in most papers and discussions of the effect of inhibitors on RSV. That is the necessity of the helper virus, RAV, for RSV maturation. Since all of these studies have relied upon the focus assay for mature RSV particles, they have completely omitted any study of RAV infection or release. Theoretically, one might say that the effect of the inhibitors on RAV is being determined if one measures their effect on RSV, as RAV determines whether or not RSV matures. However, RSV infection, uncoating, genomic replication and cellular transformation are independent of RAV (Hanafusa et al., 1964a). Therefore, the only RAV event which can be measured in these experiments is the final maturation of the virus. Work will have to be done to determine the effect of inhibitors on each of these viruses separately before virus replication can be understood. In doing this, one is faced with the problem of obtaining virus stocks, as it is easy to obtain RSV-free cultures of RAV (Hanafusa et al., 1963), but to study RAV-free RSV would require a non-defective strain of RSV. Such strains, however, are available (Results).

INTRODUCTION TO THE PROBLEM

There exists among the various strains of RSV differences including host range, serological relationships, and defectiveness (Genealogy of RSV). In order to obtain some idea of the extent of these differences, 2 strains of RSV, representing 2 biological extremes, were chosen for comparative studies. The 2 strains selected were the Bryan high-titer strain, RSV(B), and the Schmidt-Ruppin strain, RSV(SR). The Bryan strain has been the most extensively studied of all the strains of RSV and therefore was used as a comparative standard. The Schmidt-Ruppin strain has been much less extensively studied, and its ability to infect mammals and mammalian tissues, including human fibroblasts (Björn and Pontén, 1964), suggest that it possesses biological properties different from those of RSV(B).

The purpose of the following work was, therefore, to compare the biological properties of these 2 strains of RSV. Properties indicative of structural and compositional differences in the viral particles such as serology and stability under tissue culture conditions were studied. Also properties which would reveal differences in the functioning of the viral genome within the cell such as pathogenicity, defectiveness, susceptibility to RAV interference, normal virus growth and release from cells, and the effect of the inhibitors puromycin dihydrochloride and actinomycin D on viral replication were examined. Although there were definite similarities between the 2 viruses, sufficient diversity was found to warrant only cautious generalizations to other strains of RSV.

MATERIALS AND METHODS

Preparation of Water

All water used in preparing the tissue culture medium or solutions which were to come in contact with tissue cells was double distilled; that is, water obtained from a Barnstead still was distilled a second time in glass.

Preparation of Glassware

Immediately after use, all glassware was submerged in a weak Purex solution until it was to be washed. For cleaning, all glassware was boiled for 15 minutes in a 1 per cent solution of 7X (Linbro Chemical Co., New Haven, Conn.) and allowed to stand until the water was cool enough that the glassware could be handled with bare hands. The glassware was then brushed vigorously. Each piece was rinsed 10 times in running, warm tap water and 3 times in distilled water.

After it had dried, all glassware was capped or covered with aluminum foil and sterilized in a hot air oven. Syringes and pipettes were treated as other glassware except that they were cleaned by autoclaving for 30 to 60 min. while submerged in a 1 per cent solution of 7X. Syringes were then rinsed by hand as above. Pipettes were rinsed overnight in a pipette rinser and then rinsed 3 times with distilled water and allowed to dry. After drying, pipettes and syringes were placed in Dispo-wrap pipette or syringe bags and autoclaved. After autoclaving, they were dried at 60 C for at least 6 hrs.

Preparation of Tissue Culture Medium

Medium 199 without purines or pyrimidines was used exclusively. A 10 times concentrated solution of 199A (199A 10X) was prepared according to the procedure of Vogt (1963a) as given in Table I. The solution was then filtered through an O2 Selaş filter and stored at 4°C. Prior to filtration, the 199A 10X appeared slightly turbid due to the low solubility of some of the constituents.

A 10 per cent solution of sodium bicarbonate was prepared by mixing 10 gm of sodium bicarbonate in 100 ml of water and autoclaving the mixture for 15 min.

Solution DG was prepared in 3 steps (Vogt, 1963a):

1. The following were dissolved in 700 ml of water:

1-Cysteine HCl	100.0 mg
Ascorbic Acid	50.0 mg
Glutathione	50.0 mg

2. 100 mg of vitamin A was added to 10 ml of 95 per cent ethanol.

After the vitamin A was dissolved, the solution was mixed with 100 ml of a 5 per cent solution of Tween 80 in 95 per cent ethanol.

3. The solutions prepared in steps 1 and 2 were mixed and water was added to bring the total volume to 1000 ml. The DG solution was then filtered through an O2 Selaş filter and stored frozen in 10 ml quantities.

Medium 199A 1X (Vogt, 1963a) was prepared by mixing:

199A 10X	100 ml.
Solution DG	1 ml
10% Sodium Bicarbonate	7 ml
Penicillin	2×10^5 units

Table 1. Preparation of 199A 10X

Dissolve in 200 ml of boiling water:

L-Tyrosine	0.72 gm
L-Cystine	0.36 gm

Add 1.5 ml of 1N HCl, 600 ml water and dissolve:

NaCl	144.0 gm
KCl	7.2 gm
Glucose	18.0 gm
Phenol Red	0.316 gm

Dissolve in 100 ml of water and add to the above solution:

KH_2PO_4	1.08 gm
Na_2HPO_4	1.08 gm

Dissolve in 50 ml of water and add to the above solution:

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	3.60 gm
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Dissolve in 150 ml of water and add to the above solution:

CaCl_2	2.52 gm
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Weigh into a small beaker and add to the above solution:

Niacin (Nicotinic Acid)	0.45 mg
Niacinamide (Nicotinamide)	0.45 mg
Pyridoxine HCl	0.45 mg
Pyridoxal HCl	0.45 mg
Thiamine HCl	0.18 mg
Riboflavin	0.18 mg
l-Inositol	0.90 mg
p-Aminobenzoic acid	0.90 mg
Choline HCl	0.90 mg
Vitamin E (DL- α -Tocopherol)	0.18 mg
Vitamin K (Menadione Sodium Bisulfate)	0.18 mg
d-Biotin	0.18 mg
Folic Acid	0.18 mg
d-Ribose	9.00 mg
d-2-deoxyribose	9.00 mg
$\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$	1.80 mg

Table I. Continued.

Dissolve in a solution of 0.36 ml Tween 80 in 75 ml of water and add to the above solution:

Calciferol	1.80 mg
Cholesterol	3.60 mg

Bring the above solution to a total volume of 1800 ml by adding 625 ml of water; to the final solution, add the following with constant stirring:

l-Arginine monohydrochloride	1.26 gm
l-Histidine-HCl	0.36 gm
l-Lysine-HCl	1.26 gm
dl-Tryptophane	0.36 gm
dl-Phenylalanine	0.90 gm
dl-Methionine	0.54 gm
dl-Serine	0.90 gm
dl-Threonine	1.08 gm
dl-Leucine	2.16 gm
dl-Isoleucine	0.72 gm
dl-Valine	0.90 gm
dl-Glutamic Acid	2.70 gm
dl-Aspartic Acid	1.08 gm
dl- α -Alanine	0.90 gm
l-Proline	0.72 gm
l-Hydroxyproline	0.18 gm
Glycine	0.90 gm
l-Glutamine	1.80 gm
Sodium Acetate	0.90 gm
Streptomycin	0.10 gm
Penicillin	2×10^5 units

*Mycostatin	5×10^4 units
H ₂ O	900 ml

*Mycostatin was obtained from E. R. Squibb and Sons, New York.

The 199A 1X was filtered through an 02 Selas filter and refrigerated until used.

Complete Medium 199 for growing cells was composed of the following ingredients in the designated proportions (Rubin, 1960): Medium 199A 1X - 80; tryptose phosphate broth (Difco) - 10; calf serum - 5; 2.8 per cent NaHCO₃ - 2. The calf serum was obtained from Hyland Laboratories, Los Angeles, California, and was shipped from lots of serum tested by Dr. P. K. Vogt, University of Colorado Medical School, Denver, for maximum cell growth and least interference with focus formation.

Medium 199A 2X was prepared by doubling the ingredients for 199A 1X and adding 800 ml of water.

Agar overlay medium was prepared by mixing 40 parts 199A 2X, 40 parts 1.8 per cent Difco Bacto agar in water, 10 parts tryptose phosphate broth, 5 parts calf serum, and 2 parts 2.8 per cent sodium bicarbonate. The 199A 2X, calf serum, tryptose phosphate broth and sodium bicarbonate were mixed and placed in a 45 C water bath while the melted agar cooled to 45 C. At the time the agar and 199 were at 45 C the two were mixed. Only Difco Bacto agar can be used successfully, as other more pure preparations of agar suppress focus formation.

Preparation of Tris Buffered Saline

Tris saline was prepared by dissolving the following ingredients in 3 liters of water and adjusting the solution to a pH of 7.4 with concentrated

HCl (Vogt, 1963):

NaCl	24.0 gm
Na ₂ HPO ₄	0.3 gm
KCl	6.8 gm
Glucose	3.0 gm
*Sigma 7-9 or 21 (tris)	9.0 gm
Penicillin	3 x 10 ⁵ units
Streptomycin	0.166 gm

*Sigma Chemical Co., St. Louis, Missouri

This saline was sterilized by filtration unless the antibiotics were omitted, in which case it was sterilized by autoclaving.

Preparation of Trypsin

Trypsin 1:250 was obtained from Difco Laboratories, Detroit, Michigan. A solution of 0.25 per cent trypsin in tris buffered saline was prepared by adding 2.5 gm of trypsin to 1 liter of tris saline and stirring the mixture constantly at 37°C for 1 hr. The solution was then filter sterilized through an 02 Selas filter and stored frozen. It was observed that trypsin thus prepared was inactivated when allowed to remain at room temperature for 4 to 8 hours. Therefore, care was taken to freeze the trypsin immediately after use.

Preparation and Testing of Chick Embryo Fibroblasts

The eggs used throughout this research were obtained from the Montana State College poultry farm. They were incubated with the large end up at 99.9°F in a water saturated atmosphere. Primary cultures of chick embryo fibroblasts were prepared following a modification of the procedure described by Cunningham (1963) from eggs incubated 9 to 11 days. The large

end of the egg was sterilized with a 1 per cent solution of iodine in ethanol. The swabbed end of the egg was then cracked and the shell removed from over the air space with sterile forceps. The underlying membranes were peeled away with a second pair of sterile forceps. The embryo was removed by grasping it around the neck with a pair of sterile curved forceps. The embryo was then placed in a sterile 100 mm petri dish and its head and viscera removed. If the embryo was hemorrhagic or deformed, it was discarded. The eviscerated embryo was then forced through a 10 ml syringe into a 125 ml Erlenmeyer flask containing 25 ml of tris buffered saline and a Teflon coated stirring bar. The contents of the flask were stirred rapidly for about 2 minutes to free as many blood cells as possible. After 2 minutes the chunks of embryo were allowed to settle out of suspension, and the supernate was decanted. Twenty-five ml of 0.25 per cent trypsin was then added to the flask and the contents digested while being stirred rapidly for 20 min. The large chunks were allowed to settle out after this time, and the supernate was decanted through a double layer of sterile cheese cloth into a centrifuge tube containing 10 ml of medium 199. The cells were then centrifuged at 3200 r.p.m. in an International clinical centrifuge for 15 min. The supernate was removed by aspiration and discarded. The cells were suspended by vigorous pipetting in 20 ml of medium 199 and filtered again through a double layer of cheese cloth. Three ml of this final cell suspension was then plated into 100 x 20 mm Falcon plastic tissue culture dishes containing 10 ml of medium 199. The cells were incubated at 37°C in an atmosphere of 5 per cent CO₂ in air. Whenever handling

chick embryos or chick fibroblasts which were to be used to grow RSV, care was taken not to mix or in any way cross contaminate the cells of one embryo with those of another because of possible viral (Rubin, 1960a) resistance. Therefore, all materials for each embryo were handled separately.

After the primary chick fibroblast cultures were incubated for 2 days, the medium was removed and 10 ml of fresh medium 199 was added to each plate; 24 hours later, secondary cultures were prepared in the following manner. The medium was aspirated off the cells, again keeping the materials used for the cells from one embryo separate from those used for another. Four ml of 0.25 per cent trypsin was placed in each petri dish, and the plates incubated at 37.0° for 15 to 20 min. After this time, the cells were dispersed by gentle aspiration through a 5 ml pipette and the suspension placed in a centrifuge tube containing 8 ml of medium 199. The cells from plates of the same embryo were pooled at this point. The cells were centrifuged at 3200 r.p.m. in an International clinical centrifuge for 15 min. After the supernate was removed the cells were suspended in 20 ml of medium 199 and counted microscopically using a hemocytometer.

For each embryo a set of secondary cultures was prepared which consisted of (a) two 60 mm petri dishes with 5 ml of medium and 10^6 chick embryo fibroblasts per plate; (b) two 100 mm petri dishes with 10 ml of medium and 4×10^6 chick embryo fibroblasts per plate; and (c) two 100 mm petri dishes with 10 ml of medium and 6×10^6 chick embryo fibroblasts per plate. The 60 mm plates were used for testing the embryo for resistance, either viral or genetic, and the larger plates were used either for experimentation

on for viral titration. The plates with the 6×10^6 chick embryo fibroblast initial inoculation were ready for use or transfer within 4 days; however, the plates with an inoculum of 4×10^6 chick embryo fibroblasts were not ready for use before 7 days.

The procedure used for testing the resistance of embryos to virus infection was that of Rubin (1960). Immediately after plating, the 2 small petri dishes from each embryo were inoculated with RSV(B). One plate from each set was inoculated with 1000 to 1500 focus forming units (FFU) of virus, and the remaining plate from each set was inoculated with 100 to 300 FFU of virus. After incubation overnight, the supernate was removed from each plate and the medium replaced with 5 ml of agar overlay medium (see above). After the medium had solidified, the plates were again incubated. On day 4 after infection, the plates with 1000 to 1500 FFU of virus were removed and the number of foci estimated by microscopically scanning 20 per cent of the area of the plate. If there were less than 1000 foci on the plate, the embryo was judged resistant and discarded after the number of foci had been recorded. If there were 1000 or more foci on the plate, the embryo was used for experimentation. On day 7 after infection, the number of foci was counted on the plate receiving 100 to 300 FFU of RSV and this number recorded.

Preparation of Mouse Embryo Fibroblasts

Mice 15 to 18 days into pregnancy were killed by cervical dislocation. A 1 per cent solution of iodine in ethanol was then poured over the dead animal. An incision was made along the length of the abdomen, being careful

not to enter the peritoneal cavity. The skin was then peeled away from the abdomen of the mouse, and the abdominal cavity was opened with a pair of sterile scissors. The uterus was removed and placed in a sterile petri dish. One end of each horn of the uterus was then cut, and the embryos forced out of the cut end. Each embryo was decapitated and eviscerated. The procedure used for dispersion, trypsinization and plating of mouse embryo fibroblasts was the same as that described previously for chick embryo fibroblasts.

Preparation and Source of Virus Stocks

The Bryan high titer strain of Rous sarcoma virus, RSV(B), was obtained from Dr. W. R. Bryan at the National Institutes of Health, Bethesda, Maryland. The lyophilized stock was designated by Bryan as CT933. One vial was suspended in 1 ml of tris saline and 0.1 ml injected into the wing web of each of six 4 week old chicks. After 9 days the tumors were peeled from the wing of the chickens. The gross pathology of these tumors seemed to resemble that described by Rous and Murphy (193a). They were soft, friable tumors surrounded and infiltrated with a translucent, viscous fluid which showed splotches of green and brown, probably due to hemorrhage. The tumors were not encapsulated. The weight of the pooled tumors was 46.23 gm.

Virus was extracted from these tumors using a modification of the technique of Rubin (1955). One part tumor cells was mixed with 10 parts of cold tris saline containing 10 per cent calf serum and $1 \mu\text{g}/\text{ml}$ of hyaluronidase. The mixture was homogenized in the cold for 1 min. in a Waring blender. The homogenate was centrifuged for 15 min. at 5000 x g in a

refrigerated centrifuge. The supernate containing the virus was decanted and stored in sealed glass vials on dry ice. Secondary cultures of chick embryos were inoculated with 0.1 ml of this virus preparation. These cells were transferred 4 times at 2 day intervals. At the time of harvesting, the cells had become large and rounded with some giant cells appearing as described by Temin and Rubin (1958).

The virus was harvested according to the procedure of Vogt (1965). The cells were scraped from the petri dishes and pooled with their supernates. The pooled cells and supernates were then placed in an ice bath and sonicated for 5 minutes in the cold using a Ratheon Model DF101 sonic oscillator. The sonicates were then distributed in 0.1, 1, 5, or 10 ml quantities into ampules which were sealed and placed on dry ice.

The Schmidt-Ruppin strain of Rous sarcoma virus, RSV(SR), was supplied by Dr. R. J. Huebner of the National Institutes of Health. The sample received was originally sent to Huebner by Dr. C. G. Ahlström from Lund, Sweden. The sample received here was Ahlström's tumor pool #16. This sample was diluted 1:10 and 0.1 ml used to infect secondary plates of chick fibroblasts. The RSV(SR) stocks were prepared exactly as the RSV(B) stocks on tissue cells. No RSV(SR) stocks were prepared from tumors.

Rous associated virus was isolated from our stock of RSV(B) by the procedure of Rubin and Vogt (1962). RSV(B) was diluted to 10^{-7} FFU/ml and 1 ml of this dilution placed on secondary chick embryo fibroblasts. Four days after infection the cells were transferred, and 4 days later, the virus was harvested following the above procedure for harvesting RSV from

tissue culture. By interference assay (Rubin and Vogt, 1962), the titer of the Rous associated virus (RAV) stock was estimated to be about 1×10^8 interference units/ml.

Methods of Virus Assay

RSV was assayed according to the procedure of Temin and Rubin (1958) as modified by Rubin (1960a). Secondary cultures of tested chick embryo fibroblasts were prepared as described previously (Preparation and Testing of Chick Embryo Fibroblasts), except that the cells were suspended to a concentration of 2×10^5 /ml and 5 ml of this suspension added to each 60 mm petri dish that was to be used. It was found that only plastic petri dishes would give satisfactory cell sheets and foci for the virus assay. The desired dilution of virus was then added in a total volume of 0.1 ml, and the plates allowed to incubate overnight. The following morning the medium was removed from each plate and 5 ml of overlay medium 199 was added to each plate. Seven days after infection the number of foci on each plate was counted using an automatic colony counter. All virus titrations were run in duplicate with the final titer being the average of 2 plates. When preparing cells for virus assay, it was found to be very important to work rapidly so that the cells were not out of the incubator more than 2 hrs. at a time.

The interference method of assay (Rubin and Vogt, 1962) was used to titer RAV. This method makes use of the fact that a cell which has been infected with RAV will neither support the growth of nor be transformed by RSV (Rubin and Vogt, 1962). Secondary chick embryo fibroblast cultures

were prepared as above and infected with 10 fold dilutions of RAV in a total volume of 0.1 ml. For each plate infected with RAV, 1 plate remained uninfected to serve as a control. After 4 days all plates were transferred, the cells in one 60 mm plate being divided between 2 new 60 mm plates. Four days later the cells were re-transferred and challenged with RSV. For each dilution of RAV, there were 4 plates, 2 which were infected with RAV and 2 which served as the uninfected controls. Each of the 4 plates was then challenged with 100 to 300 FFU of RSV. After overnight incubation, overlay medium was added. Seven days later, the plates were examined for foci. The plate containing the highest dilution of RAV which reduced the number of foci by at least 90 per cent of the control was considered the end point. The titer of RAV was then expressed as the reciprocal of this dilution.

Isolation of Transformed, Non-Producing Clones

Transformed, non-producing (NP) clones were foci which contained transformed fibroblasts that did not release any virus. These clones were selected by infecting secondary chick embryo cells with 10 or fewer FFU of RSV(B) per plate. The cells were overlaid with medium 199 containing a 1:50 dilution of anti-RSV(B) serum. On day 7 after infection, well isolated foci were picked following the procedure of Vogt (1964a). Pipettes were prepared for picking the foci by pulling the tip of capillary pipettes out to fine points. The foci were picked, while being observed under a dissecting microscope, by tearing a circle in the layer of cells around the focus with a pipette. The focus was then sucked into the pipette and transferred to a

plate containing 10^6 secondary mouse embryo fibroblasts in medium 199. Anti-RSV serum was then added to the medium to a dilution of 1:50. Since the mouse fibroblasts will not support the growth of RAV, the use of them as feeder cells helped reduce the possibility of infecting the NP cells with RAV or RSV (Temin, 1962). After 7 to 10 days, these foci were transferred. It was possible to maintain these cells for only 3 or 4 transfers (20-30 days). After this time, the cells were difficult to transfer because of the large number of giant cells present. These cells were tested for virus production after 20 transfers by inoculating secondary cultures of chick fibroblasts with 1 ml of the supernate from the clones. By following this procedure, 85 per cent of the clones picked proved to be non-producers.

An alternative method for picking, testing, and inducing foci was developed which had several advantages. First, a feeder layer was not necessary; second, 5 foci could be handled on 1 petri dish; and third, less than 0.5 ml of medium was required per focus. Small plastic cylinders were prepared which were 6 mm high and had an internal diameter of 5 mm. After sterilization in ethylene dioxide, 5 of these cylinders were placed in a 60 mm plastic tissue culture petri dish. Three ml of 2 per cent agar in tris buffered saline was then pipetted around the cylinders. Each cylinder was then filled half full with medium 199 using a capillary pipette. Foci were then picked and 1 focus placed inside each cylinder. The cylinders were then filled the rest of the way with medium and incubated at 37.0 in an atmosphere of 5 per cent CO_2 in air. Successful growth was obtained with

90 per cent of the foci picked. Foci could be maintained for 2 weeks within the cylinder if the medium was changed every 3 or 4 days. They could also be tested and induced within the cylinder. No virus diffused between the foci during this period of time.

Preparation of Antiserum

Antiserum against RSV was prepared according to the procedure of Rubin (1960a) with the exception that 10,000 FFU were used as the initial inoculum. The virus was placed in the wing web of 4 to 6 week old chicks. Tumors were evident by 5 to 7 days after injection. After the tumors were rejected or showed marked degeneration, the birds were injected with 10^6 FFU intravenously and bled to death 3 to 4 weeks later. Attempts to prepare anti-RSV(SR) serum in this manner failed.

Excellent anti-RSV sera may also be prepared by injecting 0.1 ml of RAV, or about 10^7 interference units, intravenously into week old chicks and bleeding them 4 to 6 weeks later (Vogt, 1963).

Preparation of Fluorescent Labeled Antiserum

The procedure of Vogt and Rubin (1961) was followed for the preparation of fluorescent labeled antibody. Only antisera which would neutralize 90 per cent of the virus at a 1:100 or greater dilution were used. Ten ml of saline was placed in a 125 ml flask along with a Teflon-coated stirring bar. The saline was placed in an ice bath over a magnetic stirrer and stirred for 5 min. Ten ml of antiserum was then added and the mixture stirred for 10 min. After this time, 10 ml of saturated $(\text{NH}_4)_2\text{SO}_4$ was slowly added

dropwise. The mixture of saline, serum and $(\text{NH}_4)_2\text{SO}_4$ was stirred for 30 min. The precipitated gamma globulin was then removed by centrifugation at 5000 x g for 10 min. in a refrigerated centrifuge. The sediment was resuspended in half saturated $(\text{NH}_4)_2\text{SO}_4$ and re-centrifuged. This sediment was subsequently dissolved in 10 ml of 0.05 M sodium carbonate-bicarbonate buffer, pH 8.5, and dialyzed 24 hrs. against 4 changes of 100 volumes of the carbonate-bicarbonate buffer. Fluorescein isothiocyanate on celite 10 per cent (California Biochemical Corporation, Los Angeles) was then added to the dialyzed gamma globulin to a concentration of 25 mg fluorescein-isothiocyanate per 10 ml of gamma globulin (Rinderknecht, 1962). The mixture was shaken at room temperature for 10 min. and the excess dye removed by centrifugation. The labeled antiserum was then dialyzed in the cold against 100 volumes of pH 6.5, 0.02 M phosphate buffered saline until no fluorescence could be detected in the dialyzing fluid. Ten ml of dialyzed fluorescent antiserum was then adsorbed twice for 1 hour periods at room temperature with 250 mg of liver powder and once with 10^8 normal tested chick fibroblasts. The labeled antiserum was stored frozen in the dark until used.

Liver powder was prepared by grinding 25 to 30 gm of beef liver with an equal volume of saline in a Waring blender at 4 C. The contents of the blender were then poured into a 2 liter beaker and 4 volumes of acetone were added. The precipitate was then centrifuged and washed with saline until no hemoglobin was visible in the supernate. The washed precipitate was suspended in an equal volume of saline, and 4 volumes of acetone were

added. After the suspended material had settled, the supernate was discarded, and 4 more volumes of acetone were added. This acetone suspension was then harvested using a Buchner funnel. The precipitate was washed with several liters of acetone and dried at 37°C. The liver powder was stored in a refrigerator until used.

Fluorescent Staining of Virus Infected Cells

Virus infected cells were stained with fluorescent labeled antibody according to the procedure of Vogt and Rubin (1961). Secondary cultures of chick fibroblasts were prepared and infected as previously described except that two 18 x 18 mm, number 0 coverslips were placed in the bottom of each 60 mm petri dish. The coverslips were prepared by wiping them gently with tissue paper and sterilizing them in a hot air oven. Any further manipulation; i.e., washing, only reduced the ability of cells to adhere to the surface of the coverslips. From 1 to 4 days after infection, the coverslips were removed from the petri dishes and the medium drained from their surface. They were then placed in small staining jars containing tris saline and soaked for 5 min. After this, they were removed from the jars and the tris saline drained from them. They were then placed in a water saturated chamber. The chamber consisted of a large petri dish with a water soaked piece of filter paper in the bottom and a bar of staples resting so that the coverslips could be placed on the tips of the staples. One drop of fluorescent labeled antiserum was delivered to each coverslip from a capillary pipette. The chamber containing the coverslips was rotated so that the serum coated the entire surface of the coverslips. The chamber and

coverslips were incubated at 37°C for 10 min. The coverslips were removed from the chamber and washed for two 5 min. intervals in fresh tris saline. The coverslips were then mounted on microscope slides no greater than 1 mm thick, using a mounting fluid consisting of 5 per cent 0.1 M phosphate buffer (pH 7.0) in glycerol. The slides were examined for fluorescent staining under a Leitz SM model microscope equipped with a dark-field condenser and an ultra-violet light source. The BG12 filter plus the Streuscheibe filter were used between the light source and the condenser. The orange OG1, 2.5 mm barrier filters were used in the eye pieces. With the trinocular head, there was not enough light allowed through the monocular arm for photographs to be taken using this microscope.

The fluorescent labeled serum was tested for specificity (1) by staining non-infected chick fibroblasts, (2) by blocking the surface antigen on infected cells by a 10 min. pre-treatment of the cells with specific non-fluorescent antiserum, and (3) by attempting to stain infected cells with labeled normal chicken serum.

Preparation of Puromycin Stock Solutions

Puromycin dihydrochloride was obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio. Stock solutions were prepared by dissolving the puromycin in complete 199 to a concentration of 100 µg/ml. These solutions were stored frozen and were thawed just before use and refrozen immediately.

Preparation of Actinomycin D Stock Solutions

Actinomycin D was a gift of Merck, Sharp and Dome Research Laboratories, West Point, Pa. Actinomycin D was dissolved in 50 per cent redistilled ethanol in double distilled water to a concentration of $100 \mu\text{g/ml}$ (Vigier and Goldé, 1964). The actinomycin D was stored in the dark at -20°C and diluted to the desired concentration just prior to use.

Processing of Cells for Scintillation Counting

After the incorporation of H^3 -Leucine, cells were trypsinized and removed from suspension by centrifugation. The resulting pellet of cells was suspended in tris-saline and recentrifuged. This pellet of washed cells was suspended in 1 ml of cold tris-saline, and to this suspension, 1 ml of cold 1M trichloroacetic acid (TCA) was added. The cells immediately formed a white precipitate which was washed 3 times with 2 ml of cold 1M TCA. Between each washing, the precipitated cells were centrifuged to minimize loss of cellular material. After the third TCA washing, 4 ml of a solution of absolute ethanol:ether, 3:1, was added to the precipitate. Following centrifugation, ether was added to the precipitate. After separating the precipitate from ether by centrifugation, the precipitate was placed at 56°C for drying. The dried material was digested in 0.5 ml of 1N NaOH in a boiling water bath for 30 min. One-tenth ml was then placed in a counting vial containing 20 ml of scintillation fluid. A control consisting of 0.1 ml of 1N NaOH in 20 ml of scintillation fluid was used for background counting.

The scintillation fluid was designed for the suspension of aqueous solutions and consisted of:

1,4-Dioxane	360 ml
Toluene	360 ml
Absolute Ethanol	216 ml
2,5-Diphenyloxazole (PPO)	5 gm
Naphthalene	80 gm

RESULTS

RSV Tumors in Chickens

Virus was injected into the wing web of chickens as previously described (Materials and Methods). Figure 1 shows an intact RSV(B) tumor 14 days after injection of the virus. The tumor measured 9.5 by 5 cm. Tissue within the tumor was discolored. The tumor was soft and friable as discussed previously (Materials and Methods). The general condition of the chicken was very poor. The bird exhibited considerable weight loss and somnolence as well as loss of comb color as described by Rous (1911b). Upon post-mortum examination, the liver exhibited discoloration and contained large yellow blotches characteristic of virus involvement (Rous, 1911b).

In contrast to this marked pathology, birds challenged with RSV(SR) developed tumors much more slowly. Four weeks was required for tumors to reach a size of 2 x 3 cm, and many remained the size of a hazel nut. These tumors regressed more frequently than did RSV(B) induced tumors; however, if they did not regress, the bird eventually became ill, exhibited weight loss, and died.

RSV Infections in Tissue Culture Cells

Figures 2 and 3 show typical RSV(B) and RSV(SR) foci respectively. The cells were infected and overlaid as previously described (Materials and Methods). After incubation for 7 days, they were stained for photographing by placing 3 or 4 drops of an 0.15 per cent aqueous solution of neutral red directly on the overlay agar. After 2 to 2½ hrs., enough of

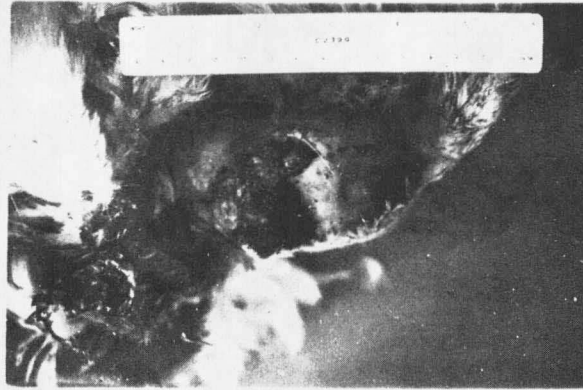


Figure 1. RSV(B) Tumor 14 Days after Infection.

the stain had been taken up by the cells to allow clear distinction of the foci from the surrounding monolayer. For routine counting, the foci were usually not stained. As may be seen in Figure 2, RSV(B) foci were small, distinct piles of cells on the monolayer; whereas, RSV(SR) foci were large, diffuse centers of infection which were not always easily distinguished from the monolayer (Figure 3).

Figure 4 is a picture of an RSV(B) focus magnified 30 times. Note the clump of rounded cells which have lost contact inhibition (Vogt, 1963). Frequently these clumps of cells would pull away from the rest of the monolayer, as this one has done, and eventually float away under the overlay medium leaving a plaque. Notice also that around the edge of the focus it is possible to distinguish the deeply stained, large, rounded, virus infected cells from the uninfected cells of the monolayer. In contrast,

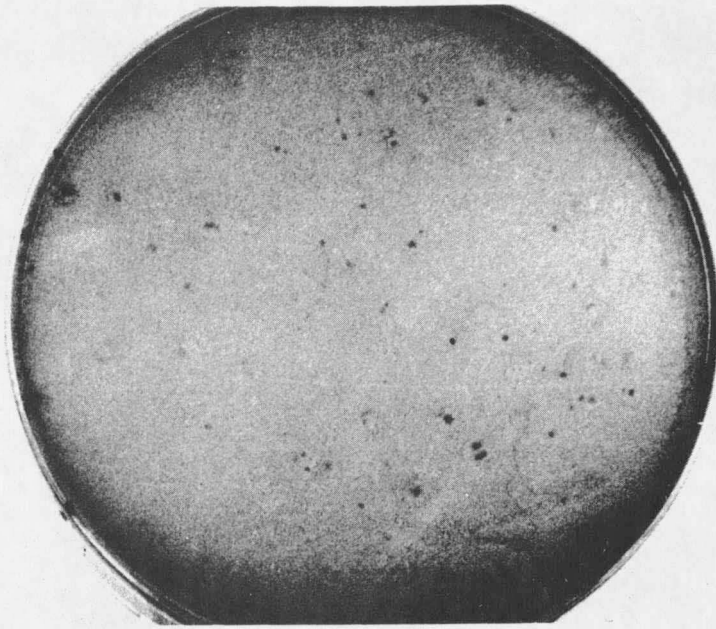


Figure 2. RSV(B) Foci.

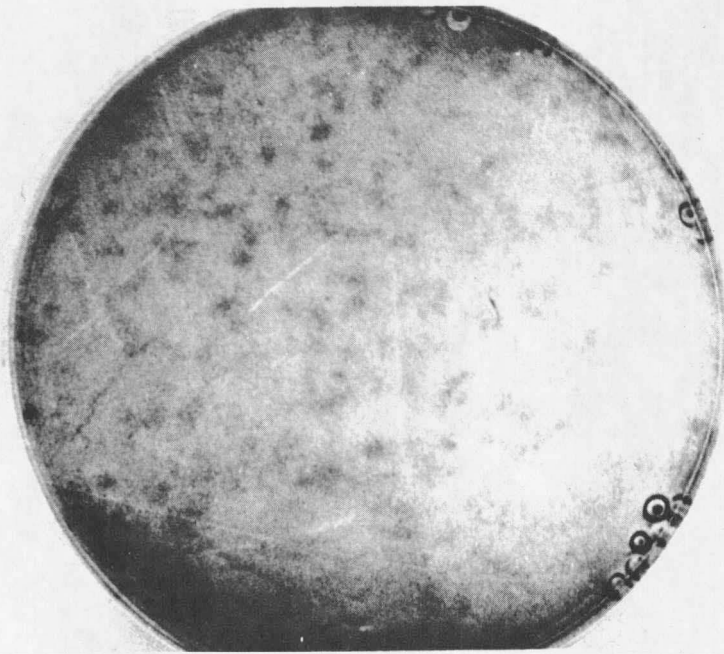


Figure 3. RSV(SR) Foci.

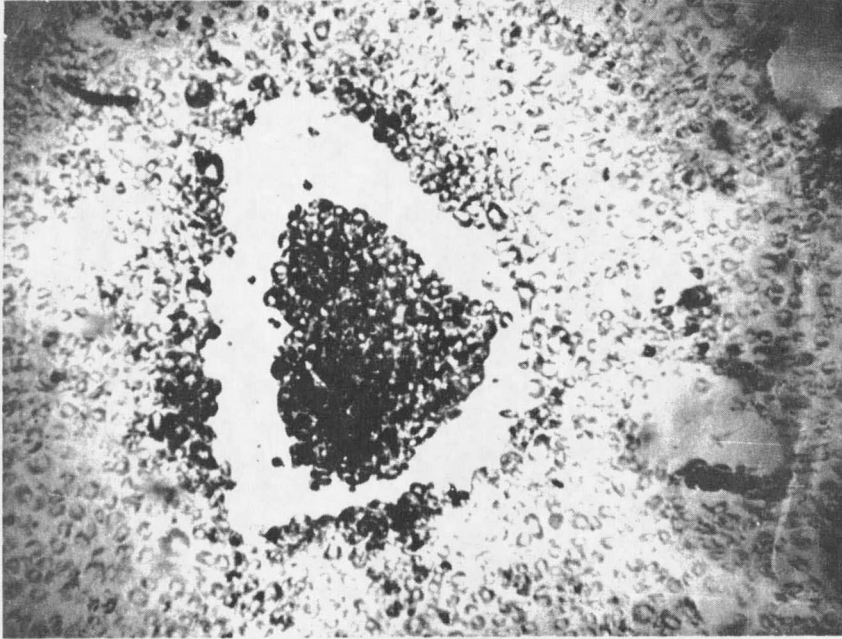


Figure 4. RSV(B) Focus Magnified 30X.

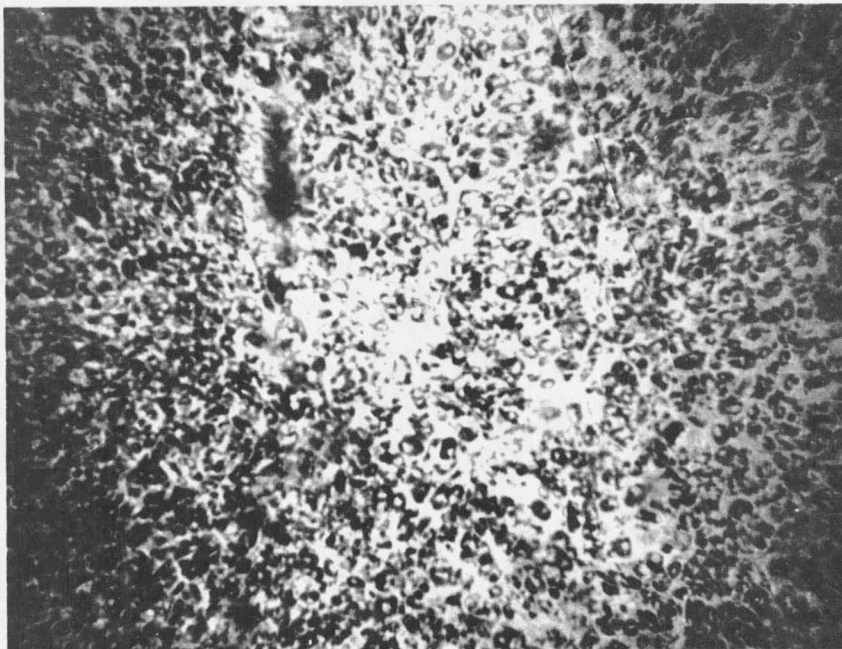


Figure 5. RSV(SR) Focus Magnified 30X.

Figure 5 shows an RSV(SR) focus under the same conditions. It was impossible to distinguish the cells of the focus from those of the monolayer. In fact, it was impossible to count RSV(SR) foci accurately using a microscope because of the large, diffuse nature of the foci. Cells within RSV(SR) foci were frequently larger than those of the monolayer, but were seldom rounded or stacked like the cells in RSV(B) foci.

Figure 6, however, shows that RSV(SR) cells did eventually become rounded and tended to lose contact inhibition. Figure 6 is a 100 x magnification of RSV(SR) cells 20 days after infection. Here is seen the characteristic morphology of established RSV(SR) cells. Very few giant cells ever developed in RSV(SR) cultures. These RSV(SR) cells could be maintained through 16 transfers or about 90 days in tissue culture. In contrast, Figure 7 shows a picture of 20 day old established RSV(B) cells magnified 100 times. Note the large degenerating giant cell near the center of the photograph. Characteristically, RSV(B) cells first had the appearance of small, granular, rounded cells, some of which may be seen around the edges of the giant cell in Figure 7. These cells also resembled the established RSV(SR) cells of Figure 6. However, after 2 or 3 transfers, a large number of giant cells were found in RSV(B) cultures. By the seventh to tenth transfer, giant cells became the dominant cell type, and because they did not divide, cell transfer was no longer possible. Therefore, RSV(B) cells could be maintained in culture only 50 to 60 days.

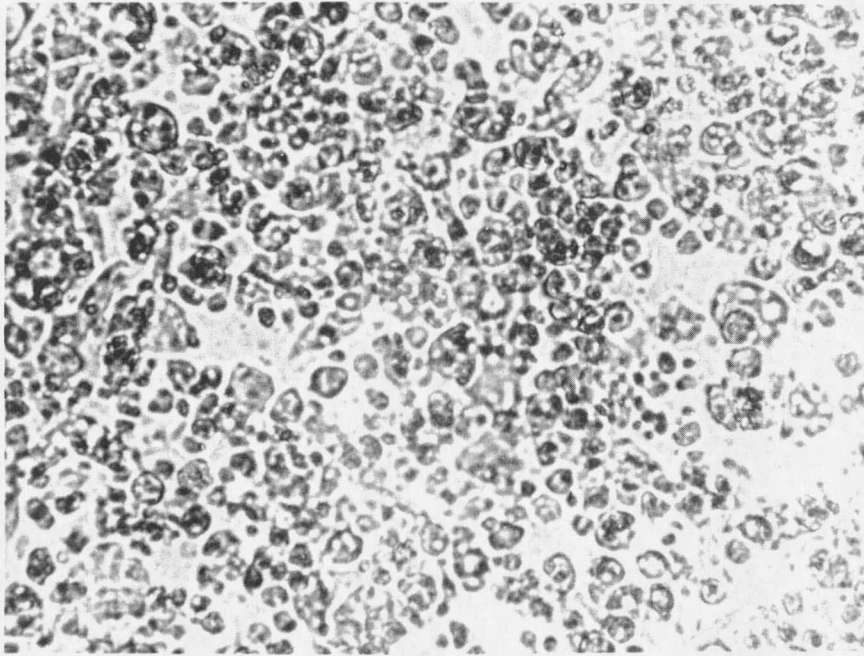


Figure 6. Established RSV(SR) Cells Magnified 100X.

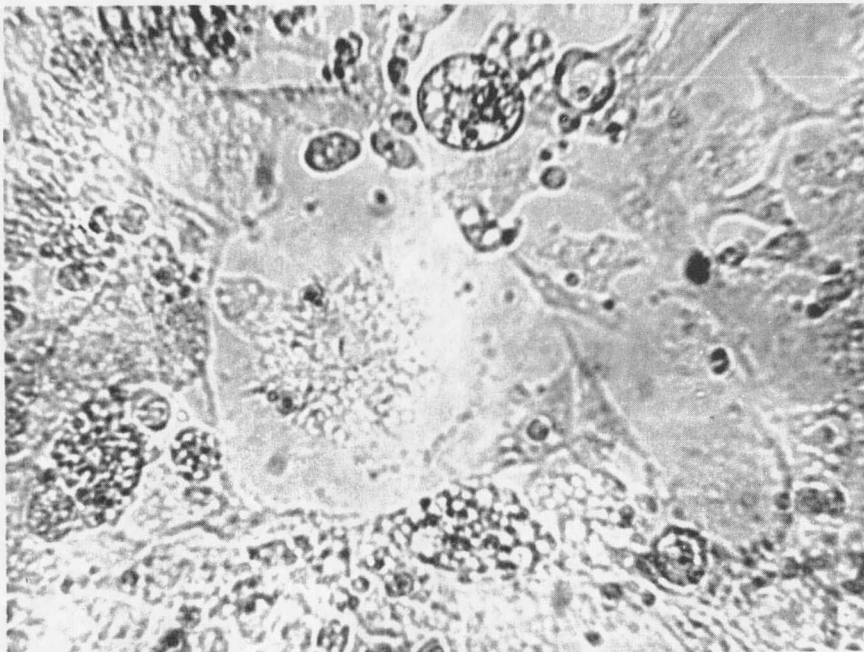


Figure 7. Established RSV(B) Cells Magnified 100X.

Heat Inactivation of RSV(B) and RSV(SR)

The purpose of these experiments was to determine the stability of each virus to tissue culture conditions. The viruses were diluted in pre-warmed, complete medium 199 and placed in a tissue culture incubator at 37°C in a water saturated atmosphere of 5 per cent CO₂ in air. At various times samples were taken and stored on dry ice until the number of surviving virus particles could be determined. Zero time was the time of addition of the virus to medium 199.

Figure 8 gives the results from these experiments. RSV(B) was more stable to tissue culture conditions than RSV(SR). The shoulder on the RSV(B) curve between 0 and 4 hrs. was probably due to virus aggregation (Hanafusa et al., 1964b). Heat inactivation constants for the 2 viruses could be estimated using an equation derived by Rubin (1955):

$$\frac{V_t}{V_0} = e^{-Kt}$$

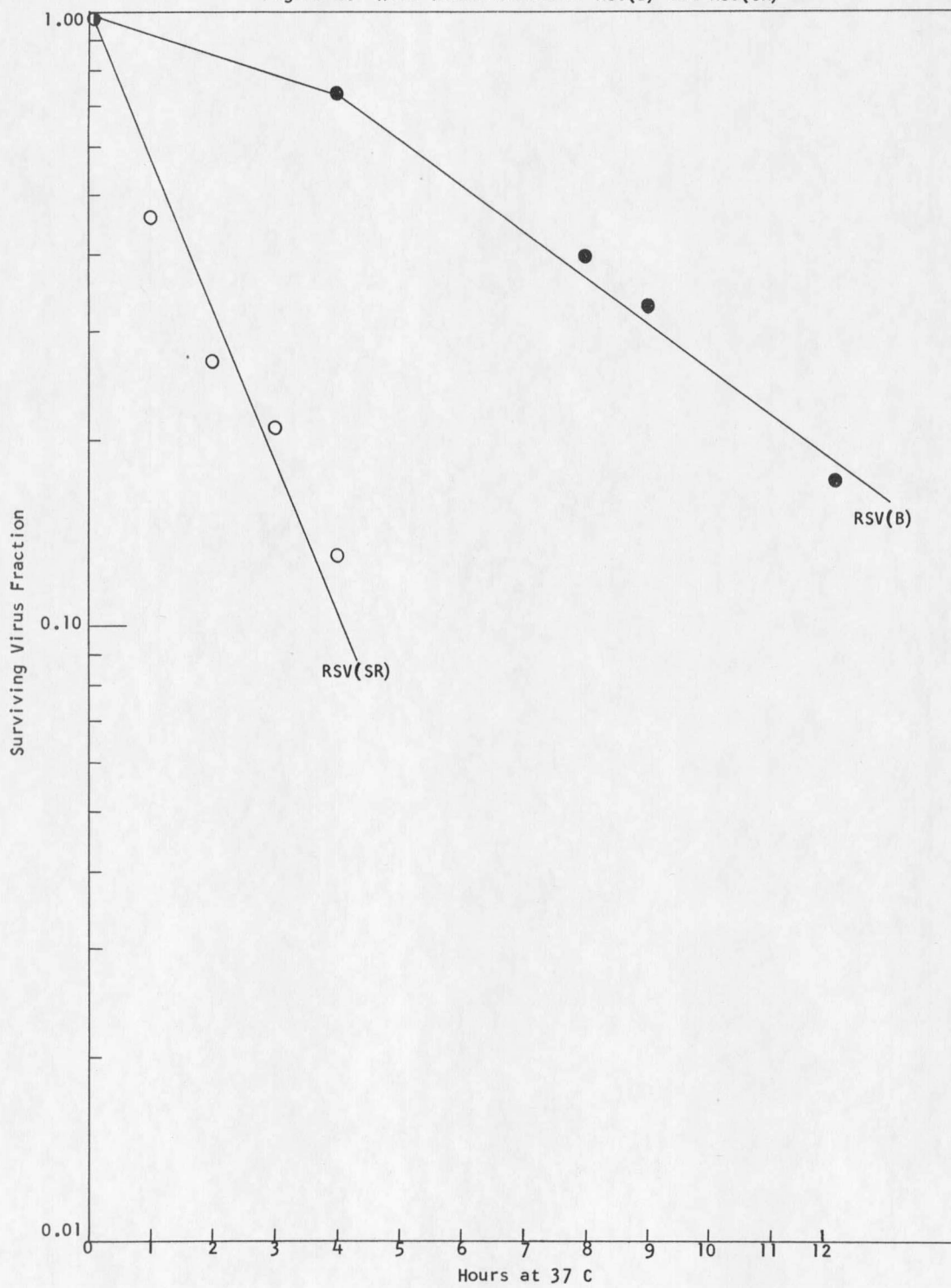
- V_t = number of virus particles surviving at time t
- V₀ = number of virus particles at time 0
- t = time in minutes at 37°C
- K = heat inactivation constant

Substituting the appropriate data from Figure 8 into this equation, the heat inactivation constant for RSV(SR) (K_{SR}) was found to be 8.85 x 10⁻³ min.⁻¹. The heat inactivation constant for RSV(B) (K_B) between 4 and 12 hrs. was 2.96 x 10⁻³ min.⁻¹. The assumption was made that this portion of the RSV(B) curve represented the inactivation of single RSV(B) particles. The K_B of RSV(B) between 4 and 12 hrs. was found to be nearly 3 times greater than the K_B between 0 and 4 hrs., indicating that, initially, the inactivation of clusters of 2 to 3 virus particles was being measured.

Figure 8. Heat Inactivation of RSV(B) and RSV(SR).

Open circles represent data for RSV(SR); closed circles represent data for RSV(B). Each point is the average of 2 experiments.

Figure 8. Heat Inactivation of RSV(B) and RSV(SR)



By setting V_0 equal to 2 and V_t equal to 1, the half-life of each virus could be calculated using the above equation. The half-life of RSV(B) was found to be 234 min. and that of RSV(SR), 78.4 min. Therefore, under these experimental conditions, RSV(B) was nearly 3 times as stable as RSV(SR).

Antigenic Relationship Between RSV(B) and RSV(SR)

A study was made of the antigenic relationship between RSV(B) and RSV(SR) using serum neutralization tests and fluorescent antibody staining of infected cells. Since the loss of infectivity of a virus due to treatment with antiserum probably results from the interaction of antibodies with those antigens on the virus responsible for adsorption to the cell and subsequent release of viral nucleic acid (Rubin, 1957; Granoff, 1965), the serum neutralization test would involve a limited number of antigens. The fluorescent antibody staining would not be limited to these few antigens, and thus should have a better chance of detecting whatever common antigens might exist on the 2 viruses. However, if the viruses have only a few antigens in common, and these few are not involved with infectivity, they would not be detected by these experiments because the amount of labeled antiserum which could be adsorbed to a cluster of viruses would be too limited to be detected by fluorescent microscopy.

Serum neutralization tests were performed following the method of Friesen and Rubin (1961). Antiserum heated to 56°C for 30 min. and prepared as previously described (Materials and Methods), was diluted in medium 199 and warmed to 37°C. A known quantity of virus was placed in the diluted

antiserum and incubated for 40 min. After this time, the amount of active virus was determined by focus assay (Materials and Methods). The control consisted of virus diluted in 199 and incubated for 40 min. at 37°C.

The results of such an experiment are shown in Figure 9, where the fraction of non-neutralized virus is plotted against the dilutions of antiserum. There was obviously no neutralization of RSV(SR) by this serum, even at dilutions which inactivate 99 per cent of the RSV(B) present. Therefore, the RSV(B) antigens which were responsible for infection were not the same as those present on RSV(SR).

RSV(B) and RSV(SR) infected cells were then stained with fluorescent labeled anti-RSV(B) serum (Materials and Methods). Serum which gave good fluorescence on RSV(B) cells gave no fluorescence on RSV(SR) cells, again showing no antigenic relationship between the 2 viruses.

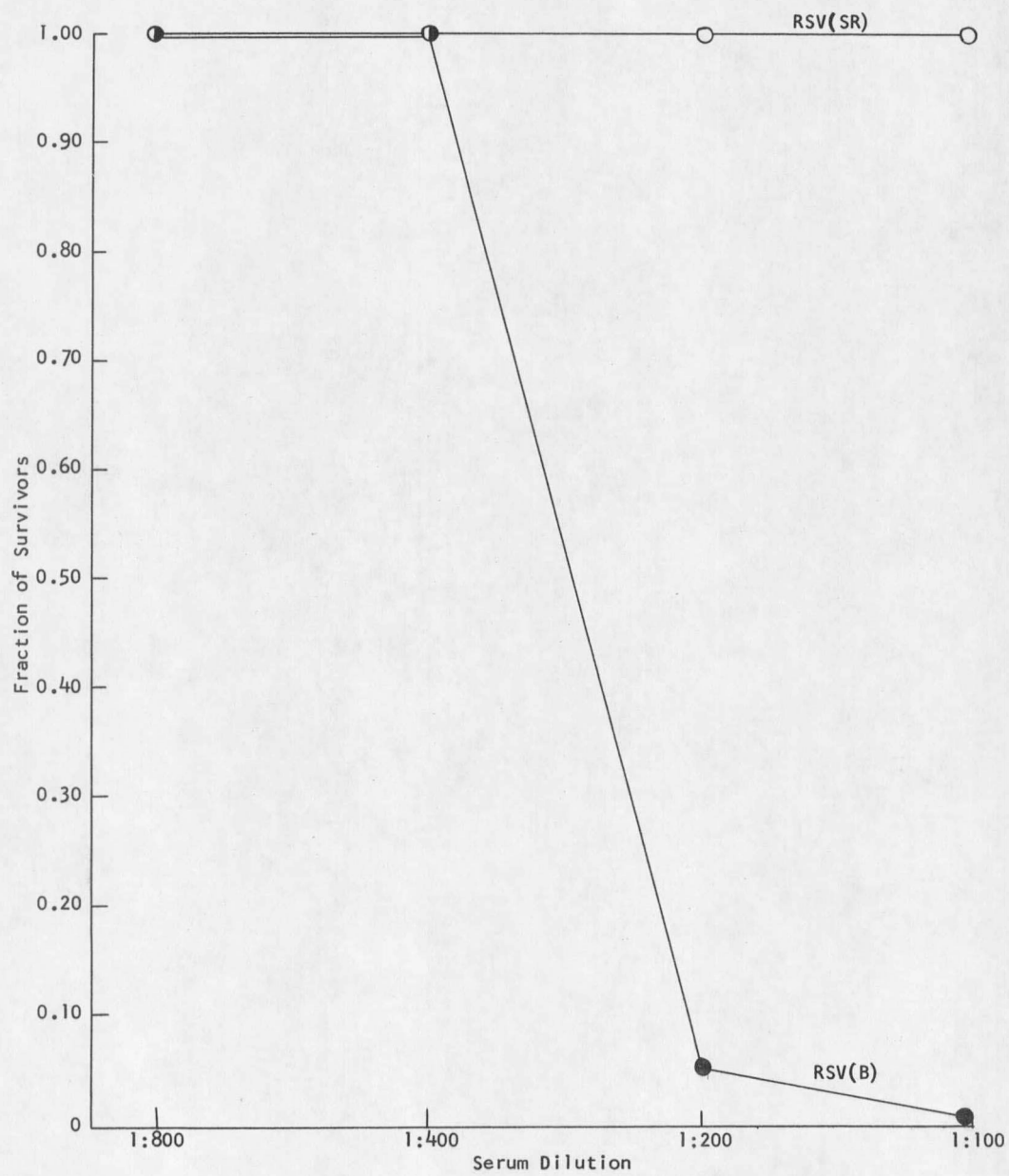
Vogt (1964b) has shown that RSV(SR) may be neutralized by antiserum against a strain of RSV(B) other than that used in this laboratory. However, Huebner et al., (1964) have found a soluble antigen in RSV(SR)-induced tumors from both hamsters and chickens which stimulates complement fixing antibodies that cross react with RSV(B) tumor and viral antigens. This antigen seems to be a group specific soluble antigen, as it has been found in all avian leukosis viruses tested.

Unfortunately, due to either the poor antigenicity of RSV(SR) or the limited amount of antigen available, it was not possible to test for RSV(B) neutralizing antibodies in anti-RSV(SR) serum. Such a test would be highly desirable since Andrews (1933) has shown that viruses exist within the

Figure 9. Neutralization of RSV(B) and RSV(SR) with Anti-RSV(B) Serum.

Open circles represent the surviving fraction of RSV(SR), and the closed circles represent the surviving fraction of RSV(B). Half filled circles are points where RSV(B) and RSV(SR) values are identical.

Figure 9. Neutralization of RSV(B) and RSV(SR) with Anti-RSV(B) Serum



avian leukosis group which will induce RSV neutralizing antibodies, but which are not themselves neutralized by anti-RSV serum.

Defectiveness of RSV(B) and RSV(SR)

Hanafusa et al. (1963) showed that RSV(B) was defective. Since many of the properties of RSV(B) were shown to be determined by the helper virus (Hanafusa et al., 1964; Rubin, 1964; Vogt, 1965; Hanafusa, 1965), experiments were carried out to test for defectiveness in RSV(SR). For RSV(SR) to be defective, it should depend upon the presence of a helper virus, i.e., RAV, for maturation, and therefore form non-producing (NP) foci. Experiments were designed to test for each of these properties.

If the helper virus which might be carried by RSV(SR) were like RAV, it would produce no morphological alterations or cytopathogenic effect on infected cells; hence, the most sensitive test for the presence of a helper virus would utilize its ability to induce virus production in NP clones. The infection of even 1 NP cell with the helper virus would lead to the replication and maturation of both the helper virus and the defective virus. After several cycles of replication, the presence of a very small number of helper viruses would result in the production of a large number of helper and defective viruses. The presence of a helper virus would then be evidenced by the presence of focus-producing, mature RSV in the supernate of cells which previously gave rise to no RSV.

An experiment was designed to test for the presence of a helper virus in RSV(SR) by induction. Several NP foci were picked from RSV(B) infected cells as described in Materials and Methods. After 2 transfers, 0.5 ml of

the supernate from the NP cells were tested for the presence of RSV(B) and therefore, indirectly for the presence of RAV. Eight of these NP clones were selected for induction as shown in Table II. Immediately before induction, the supernate from each of these clones was again tested for RSV(B), and as evidenced by the supernate control (Table II), no virus was detected.

In case the RSV(SR) helper virus might be present in concentrations not exceeding that of RSV(SR) itself, 6 of the clones were infected with 10^4 FFU of RSV(SR) (Table II). Seventy-two hours after infection, the supernate was removed and tested for the presence of virus by focus formation. Of the 6 clones induced, 6 produced virus.

Table II. Induction of Non-Producers.

Non-Producer Number	Supernate Control	RSV(SR) 10^4 FFU	RSV(SR) 10^{-6} dilution	RAV 10^{-3} dilution
1	0 ^a	+ ^b	NT ^c	NT
2	0	NT	0	+
3	0	+	NT	NT
4	0	+	NT	NT
5	0	+	0	NT
6	0	+	0	NT
7	NT	NT	0	+
8	0	+	NT	NT
^d RSV(SR) 10^{-6} Control	0			

- (a) 0 = no virus found in 0.5 ml of the medium
 (b) + = virus found in 0.5 ml of the medium
 (c) NT = not tested
 (d) 0.5 ml of a 10^{-6} dilution of RSV(SR) was tested for the presence of RSV(SR)

The possibility also existed that any RSV(SR) helper might, like RAV, be present in very high concentrations. Therefore, 4 clones were infected

with a 10^{-6} dilution of RSV(SR) (Table II). None of these clones produced foci when tested 72 hrs. later. As a positive control to show that these clones were inducible, 2 were infected with a 10^{-3} dilution of RAV (Table II). Both of these clones produced virus after induction.

The results in Table II indicate that if RSV(SR) was defective, the helper virus was present at concentrations close to that of RSV(SR) itself. They also indicate that RSV(SR) might either multiply in RSV(B) NP cells, or serve as a helper and thus induce virus production in RSV(B) NP cells.

In order to analyze for the presence of a helper virus in low dilutions of RSV(SR), and to determine whether or not RSV(SR) could serve as a helper for RSV(B), the supernate from some of the induced clones (Table II) was diluted so as to give various numbers of foci on test plates. The foci were then picked into wells as described earlier (Materials and Methods). RSV(B) foci were picked from areas adjacent to RSV(SR) foci whenever possible in order to increase the chance of contaminating the RSV(B) focus with helper virus from RSV(SR).

Table III shows that both RSV(B) and RSV(SR) were produced by some of the RSV(B) non-producers induced with RSV(SR); that all but 1 RSV(B) focus from RSV(SR) induced clones failed to release virus; that all RSV(SR) foci produced virus even when picked from uncrowded plates; and that all RSV(B) foci which arose as a result of RAV induction yielded virus. These results lead to the following conclusions. First, like other members of the avian leukosis group of viruses (Rubin, 1964), RSV(SR) can serve as a helper for RSV(B). Second, RSV(SR) is not defective. The second conclusion is

Table III. Virus Production by Foci Obtained from Induced NP Clones.

Focus Number	Focus Morphology	NP ^d Number	Number and Types of Foci on Plate from Which Focus Was Picked	Type of Virus Produced by Focus
1	B ^a	1	6SR 11B	0 ^c
2	B	1	22SR 62B	0
3	B	6	5B	0
4	B	6	5B	0
5	B	6	8SR 164B	0
6	B	6	8SR 164B	0
7	B	6	8SR 164B	0
8	B	6	8SR 164B	0
9	B	6	8SR 164B	0
10	B	6	8SR 164B	0
11	B	1	22SR 62B	SR & B
12	B	1	22SR 62B	0
13	SR ^b	4	64SR	SR
14	SR	3	32SR	SR
15	SR	1	22SR 62B	SR
16	SR	1	6SR 11B	SR
17	SR	6	8SR 124B	SR
18	SR	5	27SR	SR
19	SR	5	27SR	SR
20	SR	5	27SR	SR
21	B	2	33B	B
22	B	2	33B	B
23	B	2	33B	B
24	B	2	33B	B

(a) B = Focus morphology corresponding to that of RSV(B)

(b) SR = Focus morphology corresponding to that of RSV(SR)

(c) 0 = No virus produced

(d) NP Number refers to the non-producer number in Table II of the induced clone whose supernate was used as a source of virus.

strengthened by the observations that no foci with the morphology of RSV(SR) were found to be non-producers, and that all RSV(B) foci derived from RAV-induced clones continued to produce virus.

RAV Interference with RSV Replication

Since defectiveness was not found to be a property of RSV(SR), an experiment was designed to test the susceptibility of RSV(SR) to RAV interference. Secondary chick fibroblasts were infected with 2-fold serial dilutions of RAV. The infected cells were transferred 4 days after infection, and again 4 days later. At this time the cells were challenged with a known amount of RSV(B) and RSV(SR). Control uninfected cultures were transferred the same as RAV infected cells and challenged with the same quantities of RSV(B) and RSV(SR).

The results of this experiment are shown in Figure 10. The highest dilution of RAV which induced interference to RSV(B) was 10^{-8} . Therefore, the titer of RAV used in this experiment was 10^8 infectious units/ml. Although there seemed to be some interference with RSV(SR) at a RAV dilution of 0.5×10^{-8} , this was of doubtful significance. Therefore, it was concluded that RSV(B) and RSV(SR) showed similar degrees of susceptibility to RAV interference.

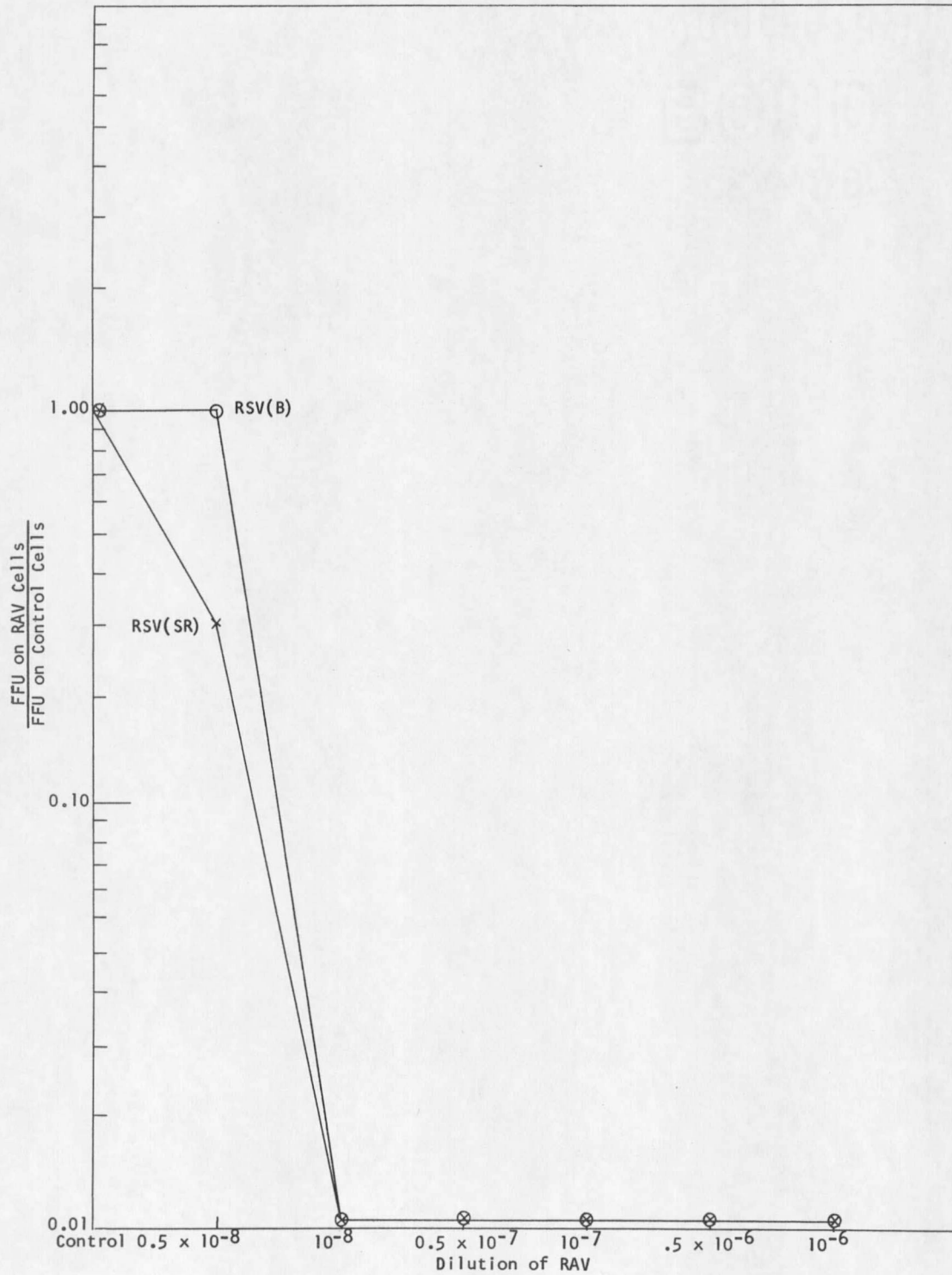
Growth of RSV(B) and RSV(SR) in Chick Fibroblasts

Eighteen to 24 hr. cultures containing 10^6 secondary chick fibroblasts were exposed to 10^6 FFU of RSV(B) or 10^5 FFU of RSV(SR) in a total volume of 1 ml for 1 hr. at 37°C. After this time, each plate was washed once

Figure 10. RAV Interference with RSV(B) and RSV(SR) Infection.

Open circles represent the data for RSV(B), and X's represent the data for RSV(SR). Each point is the average of 2 plates. The results are plotted as FFU of RSV on RAV infected cells divided by FFU of RSV on the control cells. Those points plotted as $< 0.1 \frac{\text{FFU RAV}}{\text{FFU Control}}$ are the results from cells completely resistant to infection with 4,000 FFU of RSV(B) or RSV(SR).

Figure 10. RAV Interference with RSV(B) and RSV(SR) Infection



with 2 ml of warm medium 199. Five ml of warm 199 was then added to each plate and the cultures incubated at 37 C in a water saturated atmosphere of 5 per cent CO₂ in air. Plates were removed at the designated times for sampling. The supernates from duplicate plates were pooled and stored in sealed ampules on dry ice until they were titered. The number of cells on each plate was determined and the average of duplicate samples was used to calculate focus forming units/cell (FFU/cell).

Figure 11 gives the results of such an experiment. The rate of virus release between 12 and 24 hrs. after infection can be calculated from the following equation:

$$\frac{\log V_{t_2} - \log V_{t_1}}{t_2 - t_1} = K$$

V_{t_1} = FFU/cell at time t_1

V_{t_2} = FFU/cell at time t_2

t = Time in hrs.

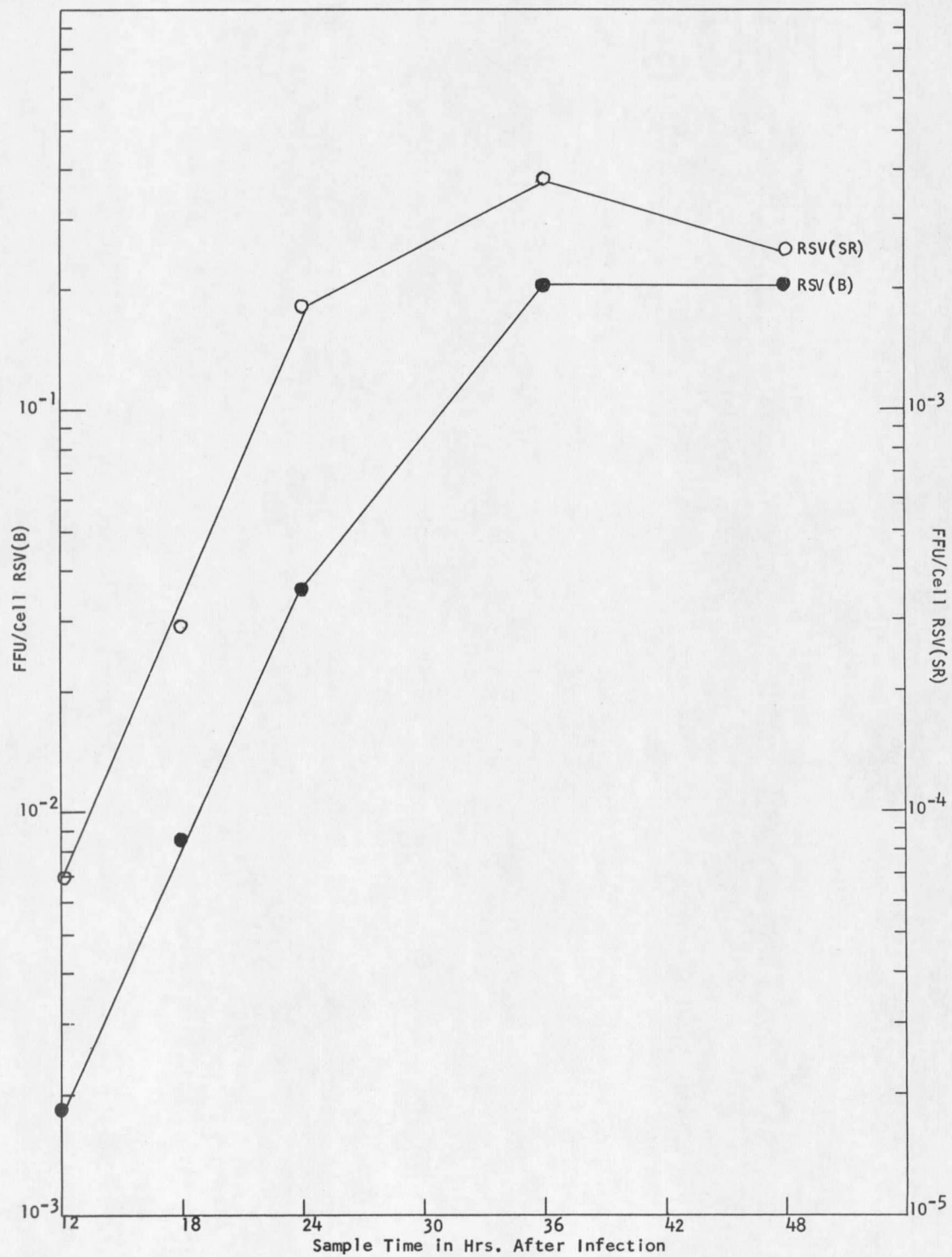
K = Rate constant for increase in virus release

By substituting values from Figure 11 into this equation, the value of K for RSV(B) infected cells was calculated to be 1.08×10^{-1} FFU/cell/hr. and K for RSV(SR) infected cells was 1.18×10^{-1} FFU/cell/hr. Therefore, virus production by both cultures increased at approximately the same rate between 12 and 24 hrs. after infection. At 24 hrs., the rate of virus production decreased for both sets of cells and finally reached a maximum at 36 hrs. after infection. RSV(B) infected cells maintained this maximum rate of virus production for 48 hrs.; however, RSV(SR) production decreased

Figure 11. Growth Curves of RSV(B) and RSV(SR).

The open circles represent data for RSV(SR), and the closed circles represent data for RSV(B). For comparative purposes the curves were drawn on the same graph. The right margin contains the ordinate values for RSV(SR), and the left margin those for RSV(B). Note the values at any point differ by 1 to 2 logs.

Figure 11. Growth Curves of RSV(B) and RSV(SR)



between 36 and 48 hrs. after infection. Indeed, as will be seen below, RSV(SR) virus production generally decreased nearly a log between 24 and 48 hrs. after infection, whereas RSV(B) production was maintained at a maximum level for up to 96 hrs.

Note the difference in the amount of total virus produced by RSV(B) and RSV(SR) at any given time. Total RSV(B) FFU was always 1 to 2 logs greater than that of RSV(SR). This discrepancy was also true of virus release from established RSV(B) and RSV(SR) cells (see below). There would seem to be something limiting the amount and not the rate of RSV(SR) production that keeps it below that of RSV(B).

Virus Release by Established RSV Cells

Established RSV(B) or RSV(SR) cells were obtained by infecting 10^6 secondary chick fibroblasts with 10^5 FFU of either RSV(B) or RSV(SR). After incubation for 4 to 5 days, the infected cells were transferred. In order to maintain established RSV cells, transfers were made every 5 to 7 days. The medium was usually changed on the third or fourth day after transfer. Established RSV cells were never used for experimental purposes prior to the third transfer.

To measure virus release by these cells, duplicate plates were prepared for each sample time. Initially, 10^6 cells were placed in each 60 mm petri dish. After 18 to 24 hrs. of incubation, the medium was removed from the plates and each washed once with 2 ml of warm 199. After washing, 5 ml of warm medium 199 was placed in the plates, and they were incubated in a water saturated atmosphere of 5 per cent CO_2 in air at 37 C (zero

time). At specified time intervals sample plates were removed from the incubator, supernates from duplicate plates pooled, and the number of cells per plate determined. Pooled supernates were stored in sealed ampules on dry ice until their virus content could be determined by focus assay.

The results of such an experiment are given in Figure 12. Both RSV(B) and RSV(SR) cells displayed a rapid release of virus between 0 and 8 hrs. RSV(B) cells appeared to reach their maximum virus release point between 8 and 16 hrs. The amount of virus released by RSV(SR) cells continued to increase, but at a much slower rate, between 8 and 16 hrs. Maximum RSV(SR) release was obtained by 16 hrs.

Both RSV(B) and RSV(SR) virus release decreased after 16 hrs. with RSV(SR) decreasing at a greater rate than RSV(B). As will be seen later, a rapid decrease in virus production after reaching a maximum level was characteristic of RSV(SR) infected cells, whereas RSV(B) virus production always decreased at a much slower rate. The 1 log or greater difference in the amount of RSV produced was also characteristic. For any given time, there was always at least 10 times more RSV(B) produced per cell than there was RSV(SR).

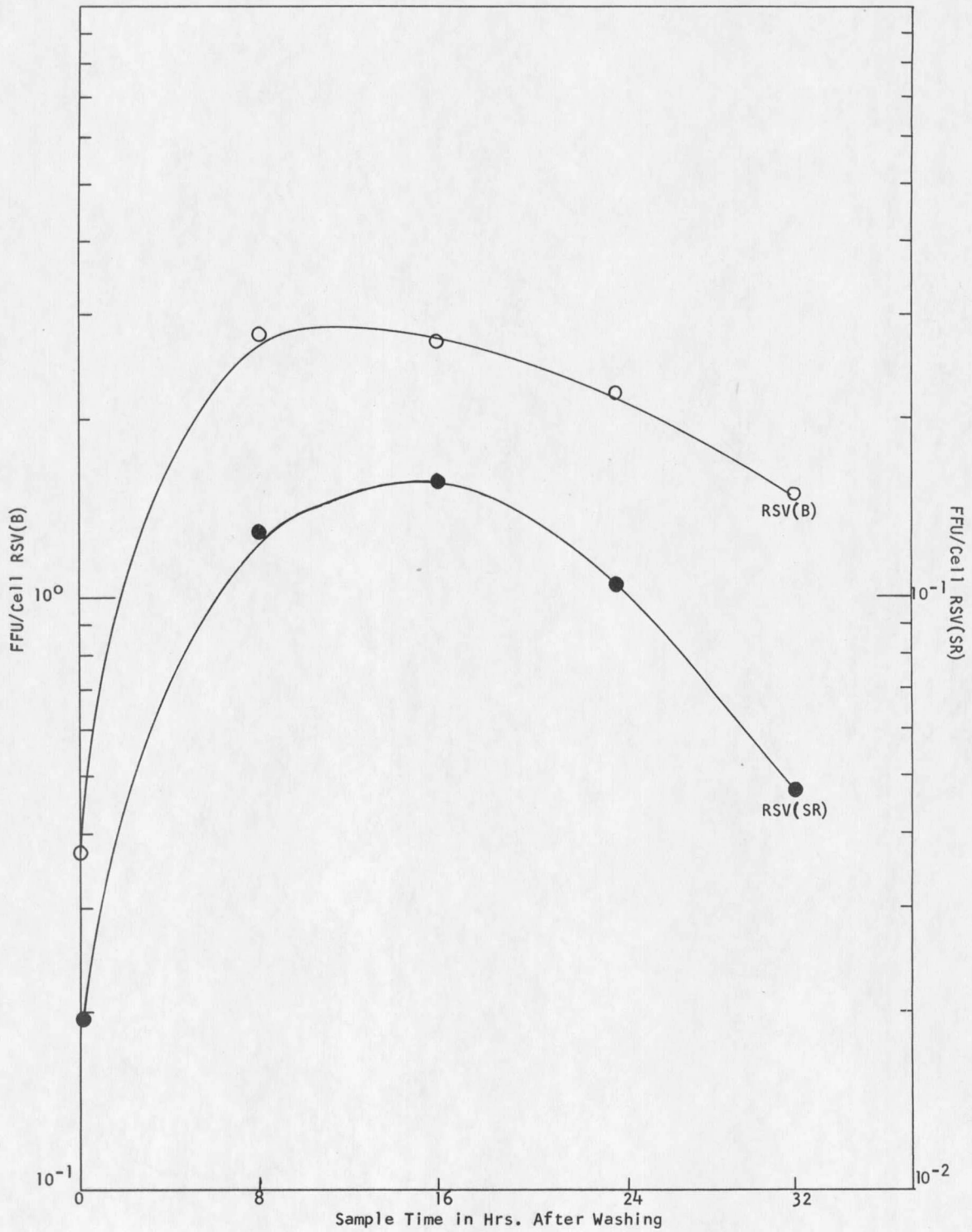
Effect of Increasing Concentration of Puromycin on Cell Division and Virus Production

When studying the effects of a metabolic inhibitor such as puromycin (PU) on a virus replicating system which requires constant viability of the cell, it is necessary to determine the concentration of the inhibitor which will produce the greatest inhibition of virus replication with the

Figure 12. Virus Release by Established RSV(B) and RSV(SR) Cells.

Open circles represent the data for RSV(B) virus release, and the closed circles that for RSV(SR). The left hand margin contains the scale for the RSV(B) ordinate, and the right hand margin contains the scale for the RSV(SR) ordinate. Each point is the average of 2 plates.

Figure 12. Virus Release by Established RSV(B) and RSV(SR) Cells



least damage to the cells. In order to do this, secondary cultures of chick fibroblasts were prepared (Materials and Methods). One set of plates was infected with 10^6 FFU of RSV(B) in 1 ml of medium 199 for 1 hr. These plates were then washed once with 2 ml of medium 199 (zero time). After this, 2 ml of 199 containing various concentrations of PU were added to each plate. For each concentration of PU, 2 plates were prepared. The virus titers were determined 24 hrs. after infection by pooling the supernates of plates containing the same concentrations of PU. A series of non-infected control plates were treated exactly as above.

Figure 13 shows that infected and non-infected cells respond similarly to increasing concentrations of PU. Maximum inhibition of cell division occurred when $2 \mu\text{g/ml}$ of PU were incorporated into the medium. At concentrations of PU greater than $1 \mu\text{g/ml}$, degenerative changes such as vacuolization and roughening of the cell surface were noted. If concentrations of PU greater than 1 to $2 \mu\text{g/ml}$ were left in contact with the cells longer than 24 hrs., the cells died and either sloughed from the plates or did not survive trypsinization.

In anticipation of extended exposures of infected cells to PU, cell counts were performed on plates exposed to $1 \mu\text{g/ml}$ of puromycin for 0 to 96 hrs. after infection. As may be seen in Figure 14, cell division was considerably retarded, and no decrease in cell numbers was noted until 96 hrs.

Figure 15 illustrates the effect of increasing concentrations of PU on virus production 24 hrs. after infection. $1 \mu\text{g/ml}$ of PU inhibited

Figure 13. Inhibition of Cell Growth by Puromycin.

Open circles represent uninfected cell counts. Open triangles represent infected cell counts. Each point is the average number of cells on duplicate plates.

