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

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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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 I. 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 I 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 considerable 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 I. 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. (1964a), 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 I 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 transmissable 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

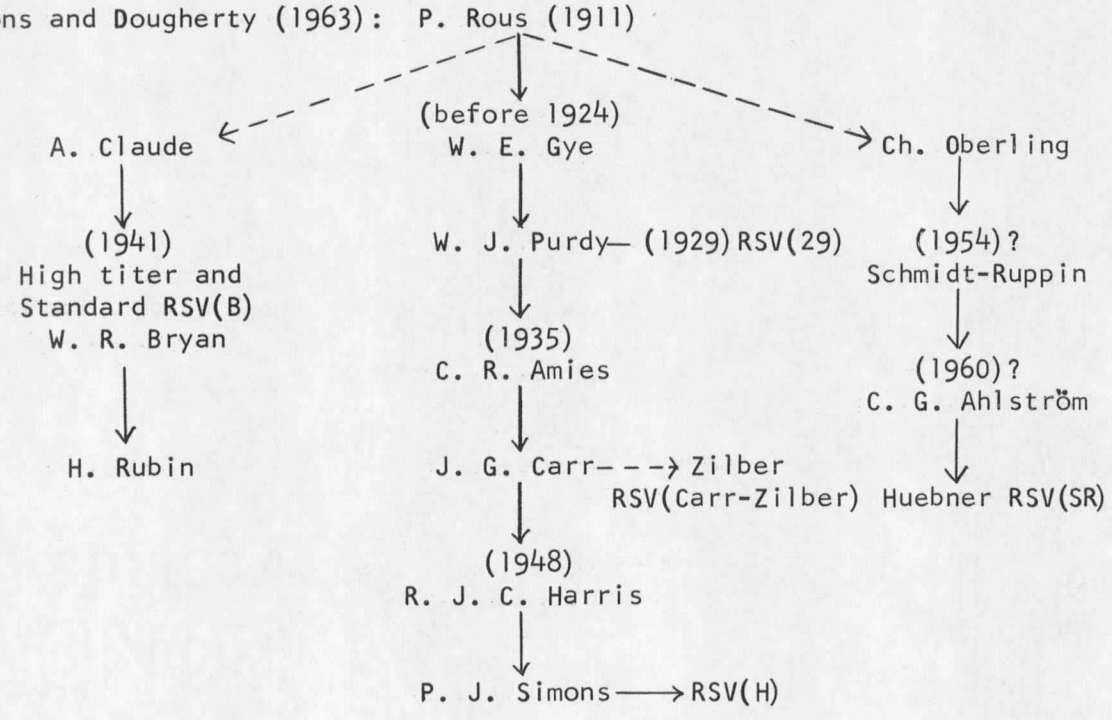
extremely fine ones, and all produced malignancies in chickens (Rous and Murphy, 1913b). Therefore, Rous and Murphy (1913b) suggested that they were all 3 of 1 class, "whatever that class may be". However, since Chicken Tumor I virus always gave rise to a spindle-celled sarcoma (Rous, 1911a) and Chicken 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):



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 chorioallantoic 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 chromosonal 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 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 Szilord, 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 μg/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 μg/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 μg/ml would stop RSV multiplication. He found that the addition of 0.2 μg/ml of AD to cells from 8 hrs. before to 24 hrs. after infection irreversibly stopped RSV production. If, however, 0.1 μg/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 nondefective 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 1. The solution was then filtered through an 02 Selas 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:

l-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 02 Selas 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 x 10^5 units