Growth Curve of Rous Sarcoma Virus and Relationship of Infecting Dose to Yield of Virus in Chick Brain 1,2

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Rous sarcoma virus (RSV) has been found to propagate in the brain of young chicks (1-3) and to kill in relation to dose (3). No gross lesions were observed in the brain, but a hemorrhagic area 0.5 to 10 mm. in diameter was almost invariably found in the meninges (3). Histologically, Rous sarcoma cells similar to those described by Vázquez-López (1) and by Duran-Reynals (2) were observed beneath the meninges but not invading the brain parenchyma (4). The data presented here show the growth curve of Rous sarcoma virus in chick brain and the relationship between the infecting dose of virus and the amount of virus recoverable from the brain.

Materials and Methods

Rous sarcoma virus (RSV), also commonly referred to as the chicken tumor I agent, was obtained from the National Cancer Institute, where it has been maintained in chickens by serial passage. Unsexed White Leghorn chicks, 3 to 7 days of age, known to be free from Salmonella pullorum, were used. Frozen, stable, standard virus was kindly provided by Dr. W. R. Bryan of the National Cancer Institute. Such standard virus was prepared by differential centrifugation from tumor tissue (5) and stored in a dry-ice chest at -50 to -70°C.

Inoculation of chicks.—Chicks were placed under light ether anesthesia and inoculated intracerebrally into the right hemisphere with 0.05 ml. of inoculum by means of a 1/8-inch 26-gauge needle. All chicks were observed daily for a period of 4 weeks and each chick was examined at the time of death for the presence of the typical hemorrhagic lesion in the meninges at the site of inoculation. Subcutaneous inoculation into the wing web was carried out as follows: Two-tenths ml. amounts were injected subcutaneously into the wing web with a 3/4-inch 26-gauge hypodermic needle. The needle was inserted through the muscle into the subcutaneous tissue of the wing web, thus avoiding leakage. Only the left wing was inoculated and the birds were examined daily for a period.

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of 3 weeks. Multiple typical tumors appeared 4 or more days after inoculation, depending upon the potency of the inoculum.

Analysis of data.—The reciprocal of time, in days, times 100 (i.e., 100/days) has been found to be a suitable transformation for analysis of both time to death following intracerebral inoculation of virus (3) and latent period for tumor production in the wing following subcutaneous inoculation of virus (6–8). Dose-response data were analyzed by the graphic methods recently illustrated in detail for tumor latent periods (6–8) and time-to-death responses (3). The potencies of all tissue suspensions are expressed as percentages relative to the observed potency of the standard, which was taken to be 100 percent in each assay. This procedure is essentially the same as that described by Bryan et al. for determining the potency of unknown tumor suspensions by tumor latent period (6–8). Differences due to fluctuation in the level of sensitivity of various test lots of chicks [see recent review by Bryan (9,10)] were therefore eliminated through reference to a standard virus within each test.

Storage of tissue and preparation of tissue extracts.—Brains were removed only from living or moribund chicks. Dead birds were routinely discarded. Each brain to be assayed was divided in half, and each half was placed in a separate ampoule, which was then sealed in an oxygen-gas flame. All such tissue was stored in the dry-ice chest at $-70^\circ$ C. until assayed. This procedure was carried out as follows: First, the brain was removed and the cerebrum was separated from the cerebellum; next, the cerebrum was divided in half transversely, so that a portion of both hemispheres was included in each half; finally, the cerebellum was divided in half longitudinally. In this manner 2 identical pools of brain tissue were made available for assay. On the day of the test the tissues were thawed and ground in a mortar with sand, and sufficient saline (containing 2% by volume of inactivated normal horse serum) was added to make a final concentration of 10 percent brain tissue by weight. This suspension was then clarified by centrifugation at 3000 r.p.m. for 20 minutes. The total time between removal of the ampoule from the dry-ice chest and the inoculation of the last chick did not exceed 2 hours.

Results

Histopathology of the brain.—It will be recalled that no gross lesions were observed in the brain, although a hemorrhagic area was almost invariably observed in the meninges in chicks dead or dying after intracerebral inoculation of RSV (3). Eyestone (4) examined histologically the brain of 1 moribund chick and observed an abundance of Rous sarcoma cells beneath the meninges but not invading the brain parenchyma. Brains were removed from 3 moribund chicks in our laboratory, and histologic examination readily confirmed the histologic picture just described. After examination of the sections, it was estimated that the number of tumor cells adherent to the brain after removal from the chick was less than 5 percent of the total weight of the brain. This observation, together with the
fact that subcutaneous tumors at the site of inoculation were never observed when standard RSV was employed and that RSV has been recovered in substantial quantities from nontumorous tissue, such as the chorioallantois (11) and liver (12) of infected embryonated eggs and from grossly unaltered liver of chickens inoculated intramuscularly with RSV (13), suggests that virus is propagating in the brain itself. However, the possibility cannot be excluded that the few tumor cells adherent to the brain after its removal may contain unexpectedly large amounts of RSV.

Growth curve of Rous sarcoma virus in chick brain.—Text-figure 1 shows the growth curve of Rous sarcoma virus in the brains of newly hatched chicks. The data on which this growth curve is based were derived from 974 chicks. The experiment consisted of 3 parts. First, 175 chicks were inoculated intracerebrally with standard Rous sarcoma virus diluted 1:10. Beginning 5 minutes after inoculation of virus, and daily thereafter, 3 chicks were killed each day and their brains removed and stored at -70°C in sealed glass ampoules. When the birds began to die as a result of inoculation of virus, brain tissue was collected each day from both living and moribund birds. Dead birds were discarded. Second, the first aliquot (see Materials and Methods) of pooled brain tissue was inoculated into the wing web of young chicks. One aliquot of each of the frozen pools of brain tissue was thawed, ground in a mortar with sand, and suspended in saline to make a 10 percent suspension by weight. The suspension was clarified by centrifugation at 3000 r.p.m. for 20 minutes. Two-tenths ml. amounts of each suspension were then inoculated subcutaneously into the wing web of groups of 10 chicks each. The birds were examined daily for a period of 14 days and the number of chicks that developed tumors in the wing was recorded. At the same time, serial decimal dilutions of standard RSV were similarly inoculated into groups of 10 chicks each. These data are summarized at the bottom of text-figure 1.

The third part of this experiment was conducted as follows: The remaining aliquots of brain tissue were thawed and ground in a mortar with sand, and sufficient saline was added to make a 10 percent suspension by weight. Each of these suspensions was then clarified by centrifugation at 3000 r.p.m. for 20 minutes. Five-hundredths ml. amounts of each suspension were inoculated intracerebrally into groups of 41 chicks each. At the same time, serial decimal dilutions of standard RSV were similarly inoculated into groups of 41 chicks each. All birds were examined daily for a period of 4 weeks.

At the conclusion of the experiment, the time to death for 50 percent of the chicks was estimated graphically for each group on probability paper by plotting cumulative mortality against time as 100/days. As anticipated, the mean time to death of chicks inoculated with standard RSV was related linearly to the dose of virus. A potency of 100 percent was arbitrarily assigned to standard RSV diluted 1:10. Thus, it was possible to estimate graphically the relative potencies of the various suspensions of brain tissue. For example, the relative potency of brain tissue collected

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on day 4 was estimated as follows: The mean time to death of the chicks inoculated intracerebrally with a 10 percent brain suspension was estimated graphically to be 6.3, expressed as 100/days, and the relative potency was then estimated graphically to be 0.14 percent. The limit of sensitivity of the method for any given experiment was determined by the susceptibility of that particular lot of chicks to RSV. Examination of the data summarized in text-figure 1 reveals: (a) Five minutes after inoculation of virus only 6 of 10 chicks inoculated into the wing with brain suspension developed tumors, thus indicating that infective virus disappeared very rapidly from the brain. (b) Very little virus could be recovered from brain tissue through the 3rd day after inoculation of RSV. (c) However, beginning with the 4th day after inoculation virus was present in relatively large amounts and increased at a regular rate until it reached its peak in moribund or prostrate birds. (d) The peak of viral potency attained was comparable to, or higher than, the standard virus used to initiate the infection. (e) Brain tissue removed from moribund birds was more potent in 2 instances than brain tissue removed on the same day from birds that appeared healthy. Utilizing the portion of the growth curve that showed a logarithmic increase in potency (i.e., between the 4th and 9th days after inoculation), it can be calculated that during this period the time to double the potency of brain tissue was approximately 9.4 hours. Since the stability of RSV in brain tissue is not known, it is impossible to estimate what might be comparable to generation time in bacteria.

Text-figure 1.—Growth curve of Rous sarcoma virus (RSV) in chick brain. ○ = Tissue collected from living chicks; ● = tissue collected from moribund chicks.
Effect of initiating dose on yield of virus.—Text-figure 2 summarizes data illustrating the relationship between the infecting dose of RSV and the amount of virus recoverable from the brain. The data collected in these

![Text-figure 2](https://academic.oup.com/jnci/article-abstract/18/4/507/967703)

**Text-figure 2.**—Relationship of infecting dose of RSV to yield of virus from chick brain. ○ = Experiment 1; ● = experiment 2.
experiments were based on 1,435 chicks. The experiment consisted of 2 parts. The first is shown in the upper portion of text-figure 2. Groups of 35 chicks each were inoculated intracerebrally with serial decimal dilutions of Rous sarcoma virus, as indicated. In addition to recording mortality in each of the groups, brains were removed from 5 moribund or prostrate chicks in each dilution group. Dead chicks were discarded. Each brain was placed individually in an ampoule that was then sealed in an oxygen-gas flame and stored at $-70^\circ$ C. The LD50 of standard Rous sarcoma virus was approximately $10^{-5.2}$. The second part of the experiment is shown in the lower portion of text-figure 2. The individual brains frozen from the first experiment were thawed and ground in a mortar with sand; then sufficient saline was added to make a 10 percent suspension by weight, which was clarified by centrifugation at 3000 r.p.m. for 20 minutes. Each of these suspensions was inoculated intracerebrally as a 10 percent suspension into groups of 35 chicks each. At the same time, 4 serial decimal dilutions of frozen standard RSV were inoculated intracerebrally into groups of 35 chicks each. The mean time to death of the various groups of chicks was estimated graphically as before and the relative potencies of the individual brains were estimated as illustrated in text-figure 1. These potencies have been plotted in the lower part of text-figure 2 against the dilution of RSV inoculated. It should be emphasized that each brain assayed was removed from a chick that was prostrate or moribund and showed the typical pathognomonic hemorrhagic area in the meninges at the site of inoculation. Examination of the data summarized in text-figure 2 reveals: (a) When chicks were inoculated intracerebrally with more than 1 LD50 of RSV, the brains contained appreciable and relatively uniform amounts of virus. (b) However, when chicks were inoculated with less than 1 LD50 of RSV, the brains contained, in 4 of 5 cases, no recoverable virus. These findings agree with and confirm those of Bryan et al., who showed that when the initiating dose of Rous sarcoma virus was less than 1 ED50 the virus extractable from a given wing tumor was either very low or could not be detected at all (14).

Discussion

Taken as a whole, the growth curve of Rous sarcoma virus in chick brain parallels that of other animal viruses. Thus, 3 phases commonly observed with other viruses are also found with Rous sarcoma virus.

These are: (a) A large proportion of the infective virus rapidly disappears from the tissue; then (b) a stationary phase or latent period occurs during which the infective-virus content of the tissue remains low, followed by (c) a progressive increase in viral concentration in the tissue. It appears that the growth curve of RSV in chick brain shows no obvious dissimilarity to that of the non-neoplastic animal viruses. Unfortunately, experimental data showing the relationship between the number of virus particles and the minimal infective dose are lacking. Consequently, little can be said regarding the stationary phase and its relationship to the so-called "eclipse phase" observed with bacteriophage and certain other viruses (15).
One of the most provocative observations in recent years was made by Bryan, Calnan, and Moloney (14), who found that the amount of virus extractable from a given tumor was directly related to the amount of virus used to initiate that tumor. Indeed, when high dilutions of virus were used, which produced tumors in less than half the chicks, 24 percent of such low-dose tumors yielded no extractable virus. The data presented in our report confirm this observation in chick brain. Failure to recover any infective virus from the brains of 4 of 5 moribund chicks inoculated with less than 1 LD50 of RSV is of considerable interest, particularly in view of the fact that the growth curve of RSV in chick brain clearly indicates that the maximum quantity of RSV is found in brain tissue removed from chicks just about to die. These data are also at variance with common experiences with many of the non-neoplastic animal viruses. In many laboratories, virus has been routinely recovered from dead or diseased animals inoculated with limiting dilutions of virus in order to establish the cause of death or disease. Although speculation concerning the nature of this phenomenon is premature at this time, it is of interest to recall the studies with the electron microscope made by Epstein (16), who observed that, at most, only 1 ascitic Rous sarcoma cell in 50 contained virus-like particles. Whether or not the infective-virus content of a given cell or tissue fluctuates with time is unknown.

Summary

The growth curve of Rous sarcoma virus (RSV) in chick brain paralleled that of many non-neoplastic animal viruses. First, virus disappeared rapidly from the brain, and 5 minutes after intracerebral inoculation of RSV, virus was barely detectable in brain tissue. This was followed by a stationary phase of approximately 3 days during which the infective-virus content of the brain remained low. Finally, beginning with the 4th day after inoculation, the viral content of the brain increased logarithmically and reached its maximum in moribund chicks. When chicks were inoculated intracerebrally with 1 to 10,000 lethal doses (LD50) of RSV and brains were removed only from moribund chicks, such brain tissue contained appreciable and relatively uniform amounts of virus. However, when chicks were inoculated intracerebrally with less than 1 LD50 of RSV, brains removed from the relatively few chicks that became moribund contained, in 4 of 5 instances, no recoverable RSV. This observation is in agreement with data on the yield of recoverable virus from tumor tissue [see Bryan, Calnan, and Moloney (14)] and is at variance with common experiences with many non-neoplastic viruses.

References


(6) **JEHSTONE, W. H.:** Personal communication cited in (3).


