TABLE I (continued).

Amino acid	pH	Kd at °C				Rf calc. at °C (× 100)				Rf published (McFarren) (× 100)
		20	25	30	22	20	25	30	22	22
Serine	2.0	8.1	7.2	7.8	7.8	19	22	20	20	23
	6.2	6.5	7.2	7.0	6.8	25	22	22	23	20
	10.0	5.8	6.1	4.5	5.9	27	26	35	27	28
	11.2	6.6	6.5	6.2	6.6	24	24	26	24	18
Threonine	2.0	7.1	6.7	6.0	7.0	22	23	27	22	35
	6.2	5.6	5.0	5.0	5.4	29	33	33	30	37
	10.0	4.7	4.5	3.9	4.6	35	38	40	35	40
	11.2	5.0	4.8	4.6	4.9	33	34	35	33	34
Tyrosine	2.0	2.0	2.0	1.8	2.0	60	60	64	60	56
	6.2	1.4	1.4	1.5	1.4	70	70	68	70	60
	10.0	1.4	1.4	1.3	1.4	70	70	72	70	61
	11.2	1.4	1.4	1.4	1.4	70	70	70	70	60
Tryptophane	2.0	.41	.43	.44	.42	89	89	89	89	82
	6.2	.27	.29	.29	.28	86	87	87	86	85
	10.0	.22	.27	.29	.24	83	86	87	84	88
	11.2	.22	.23	.24	.22	83	84	84	83	85
Valine	2.0	2.0	1.7	1.8	1.9	60	65	63	62	69
	6.2	1.7	1.5	1.6	1.6	65	68	66	67	71
	10.0	1.5	1.5	1.5	1.5	68	68	68	68	70
	11.2	1.6	1.5	1.3	1.6	66	68	72	67	72

culated from each of these distribution coefficients have been correlated by statistical methods with corresponding Rf values as measured by paper chromatography and a significant relationship has been demonstrated.

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Host-Virus Relationships in Rous Sarcoma Tissues *in vitro*. I. Growth of Extraneous Viruses.* (23693)

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Considerable experimental work has been recorded on the growth of viruses in neoplastic tissues(1). For such investigations most workers have utilized transplantable tissues of tumors not known to be caused by viruses, while few studies have been made of the growth of extraneous viruses in the cells of virus-induced tumors. In investigating the biological properties of a tumor-inducing virus and the properties of the malignant tissue it produces, the ability of Rous sarcoma tissue

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(2) to support the growth of several extraneous animal viruses has been studied. The Rous sarcoma tissue is uniquely suited for such studies, for it is of proven malignancy; it is a consequence of virus infection of normal connective tissue elements(3); and recent evidence indicates that all Rous tumor cells contain and slowly produce the Rous virus(4). Thus, by employing this particular tissue, it has been possible to study the capacity of a malignant tissue already infected with a cancer virus to support the growth of certain nonneoplastic viruses. The ability of normal chick embryo and adult chick fibroblastic tissues to support the proliferation of the same infecting viral agents has also been studied. It was anticipated that the findings from such studies might provide valuable information of several varieties. The studies of the attempted superinfection of the Rous cells were of interest for the information they might give regarding possible demonstration of an interference phenomenon or of dual virus infection of tumor cells. This information in turn might elucidate certain biological properties of the Rous sarcoma virus and thereby provide some insight as to the relationship of this virus to nonneoplastic viruses. Because of the biological and morphological relationships of the embryo and adult chick fibroblastic tissues to the chick-derived Rous sarcoma tissue, the studies of the virus proliferation in these related tissues were of interest for the clues they might provide regarding similarities or differences between these 3 tissues in their reactions to viral infection.

The results indicate that of 4 viruses capable of proliferation in chick embryo fibroblastic tissue *in vitro* (Newcastle Disease virus (NDV), the NWS strain of influenza A virus (NWS), vaccinia virus and psittacosis virus), only NDV and vaccinia virus were able to reproduce in cultures of adult chick fibroblastic tissue and of Rous sarcoma tissue.

Materials and methods. Viruses. The Rous sarcoma virus used for the induction of tumors was the partially purified CT-581 preparation(5) kindly provided by Dr. W. R. Bryan. All viruses used were stored sealed in glass in a dry-ice freezer. The 6BC strain of psittacosis virus used was passed in eggs by the yolk sac route, and a uniform source of the agent was obtained by methods described previously(11). The strain of vaccine virus employed was that used by the New York City Board of Health. To obtain a fairly uniform source of the virus, 12-day embryonated eggs were infected by the CAM route. After 72 hours' incubation, the membranes were removed, pooled, homogenized, centrifuged and diluted in infusion broth to make a 20% suspension by weight. The B strain of NDV and the NWS strain of influenza virus were prepared by harvesting the infected allantoic fluids 48 hours after inoculation of eggs, centrifuging the materials, and removing the supernatant fluids. *Virus titration.* The presence of Rous sarcoma virus in tumor cell cultures was determined by the ability of the material tested to produce characteristic lesions in young chicks. 0.20 ml of the homogenates of the cultures prepared as below were inoculated into the wing web of 6 to 8 white leghorn chicks under 10 days of age according to the method of Bryan *et al.*(5). In some cases, white leghorn chicks were inoculated intracerebrally with 0.05 ml of the homogenate as described by Groupé and co-workers (6). In determining the amount of psittacosis virus in seed stock or in culture materials, the single dilution technic of Golub was employed(7). In titration of vaccinia-infected materials, the 50% lethal endpoint was calculated according to the method of Reed and Muench(8) using the method described previously(9a,b). Stock and experimental fluids infected with NDV and influenza virus were diluted in serial 10-fold dilutions in chilled beef infusion broth and four 9-day-old embryos were inoculated allantoically with 0.20 ml of each of the dilutions. After 48 hours' incubation at 35 to 36°C, 0.5 ml of allantoic fluid was removed from each egg and the presence of virus determined by testing for hemagglutinins by the method of Granoff and Henle(10). The 50% infectivity endpoint was calculated. *Culture Media.* The culture media employed were: A. In chick embryo fibroblastic cultures, horse serum 23.0%, Lactalbumin hydrolysate 0.8%, Hanks' balanced salt sol. (BSS) 76.2%. B. In adult chick fibroblast and Rous sar-

TABLE I. Growth of NWS Influenza Virus.

Tissue	Inoculum	Virus content: 6 hr	2nd washing at 6 hr	2 days	4 days	6 days	8 days
Chick embryo	$10^{4.5}$*	$F = 10^{3.25}$	$10^{2.17}$	$F = 10^{1.76}$	$F = 10^{5}$	$F = 10^{5}$	$F = 10^{5.76}$ $T = 10^{6}$
Adult chick	$10^{4.75}$	$F = 10^{4}$	$10^{1.75}$	$F = 0$	$F = 0$	$F = 0$	$F = 0$ $T = 0$
Rous sarcoma	$10^{4.76}$ $10^{7.0}$	$F = 10^{1}$ $F = 10^{6}$	$10^{2.4}$ $10^{5.0}$	$F = 0$ $T = 0$ "	$F = 0$ $T = 0$ "	$F = 0$ $T = 0$ "	$F = 0$ $T = 0$ "
Virus controls	$10^{4.5}$	10^{4}	—	0	0	0	0
Tissue "	0	0	—	$F = 0$ $T = 0$	$F = 0$ $T = 0$	—	$F = 0$ $T = 0$

* LD_{50}. F = fluids, T = tissues, -- = not tested.

coma cultures, horse serum 40.0%, Lactalbumin hydrolysate 0.8%, BSS 59.2%. Media were buffered to pH 7.4 with 4% sodium bicarbonate and contained 100 units/ml of penicillin and 50 gamma/ml of streptomycin. No antibiotics were used in the psittacosis cultures. *Tissues.* The Rous sarcoma tissue used was taken from tumors produced in the wing web of white leghorn chicks by the subcutaneous inoculation of dilutions of the standard stock preparation in chicks under 10 days old. Tissue was removed for *in vitro* culture 10 to 14 days after inoculation from tumors which were rapidly developing and not necrotic. The malignant appearing tissue was dissected, efforts being made to avoid the inclusion of nonmalignant muscle and/or connective tissue. The adult chick tissue used was the skin, connective tissue and muscle dissected as above from the wing web area of 20- to 30-day-old white leghorn chicks. The embryo fibroblastic tissue employed was the skin, connective tissue and muscle dissected from the wings and legs of 12-day-old embryos. *Culture technic.* The tissue cultures were prepared as described previously(11), using fragments of tissues planted under cellophane discs in 10-ml Erlenmeyer flasks. The initial medium was completely removed after 24 hours' incubation at 36 to 37°C, and the tissue washed once with BSS. Each of 4 cultures was infected with a particular virus and after 6 hours' incubation at 36 to 37°C, the fluids were completely removed, pooled, centrifuged for 5 minutes at 2,000 rpm and the supernatant fluid taken for the determination of virus content. The tissue was washed twice with 2 ml of BSS, the second washing being prepared as above for virus titration. Thereafter, at 2-day intervals, the culture fluids were completely removed and prepared for titration. Each time, as the medium was removed, the tissues were washed once with BSS and fresh medium replaced. The fluids were titered for the presence of both the exogenous virus and Rous sarcoma virus in the case of the Rous sarcoma cultures. In some experiments, the tissue from one flask was removed at the time of harvest of the fluid medium and was prepared for determination of virus content. In all experiments the tissues of the flasks remaining at the end of the 8-day experimental period were pooled and prepared for virus titration. The tissue was ground in a mortar with alundum in 1 ml of BSS. The homogenate was then centrifuged for 5 minutes at 2,000 rpm and the supernatant removed for titration. Since these homogenates were tested for activity of the Rous sarcoma virus as well as the extraneous virus, careful microscopic examination was made of the material to assure that intact cells were not present.

Controls. Virus controls to determine thermal inactivation of the individual viruses were prepared by inoculating the same quantity of virus used in the experimental protocol into 10-ml Erlenmeyer flasks containing an identical quantity of the appropriate medium. These tissue-free cultures were incubated at 36 to 37°C and at periodic intervals aliquots were taken for the estimation of concentration of active virus.

Results. The results of experiments to de-

TABLE II. Growth of Psittacosis Virus.

Tissue	Virus content: Inoculum	6 hr	2nd washing at 6 hr	2 days	4 days	6 days	8 days
Chick embryo	$10^{4.8}$*	F = $10^{3.5}$	$10^{2.45}$	F = $10^{3.78}$	F = $10^{5.5}$	F = $10^{5.78}$	F = $10^{5.2}$ T = $10^{5.8}$
Adult chick	$10^{4.7}$	F = $10^{3.1}$	$10^{2.5}$	F = 0	F = 0	F = 0	F = 0 T = 0
Rous sarcoma	10^{5}	F = $10^{3.9}$	$10^{2.48}$	"	"	"	"
Virus control	10^{5}	$10^{3.15}$	—	0	0	0	0
Tissue "	0	0	—	0	—	0	—

* LD_{50}.

F = fluids, T = tissues, — = not tested.

termine the ability of the NWS type of influenza A virus to multiply in the embryo, adult and tumor tissues are summarized in Table I. Active multiplication of NWS was evident when the embryo fibroblastic tissue was used as the host tissue. During the 6-hour period allowed for attachment of the virus, concentration of the NWS recoverable from the fluids of the cultures diminished markedly. However, at 2-day intervals thereafter the concentration of the extracellular virus steadily increased, in contradistinction to that in the virus control cultures where no virus was demonstrable at any examination after the initial 6-hour titration. The only examination of the virus content of the tissues was made on the 8th day of infection and a large amount of intracellular virus was demonstrated.

In contrast to these findings, there was no NWS virus demonstrable in either the fluids or the tissues of the adult chick or Rous tumor cultures at any time later than 6 hours following the inoculation of the virus. And in the case of the tumor tissue, these findings were independent of the concentration of the NWS inoculum. Such results clearly indicate that there was no proliferation of the NWS virus in these cultures.

The tissues of both types of cultures, however, were metabolically active, as evidenced by the drop in the pH of the culture medium, and the finding of active Rous sarcoma virus in both fluids and tissue of the tumor cultures indicated that virus was present. Furthermore, no hemagglutination-inhibiting substances were found in either the medium or extracts of the tissues which might have masked the NWS virus. Thus, the inability of the NWS virus to multiply in the cultures of the adult chick and Rous sarcoma tissues *in vitro* is due to some direct relationship between the cells and the virus and not to extraneous factors.

The findings with psittacosis virus are summarized in Table II. They appear qualitatively similar to the results of the NWS studies in that there is clear evidence of psittacosis virus multiplication in the chick embryo tissue but no indication of proliferation of the virus in the adult chick or tumor tissues.

The concentration of psittacosis virus demonstrable in the medium of the embryo tissue cultures was found to increase progressively throughout the course of the experimental period with maximum titers found 6 days after infection.

No psittacosis virus was demonstrable in the fluids of the cultures of the adult chick tissue or of the Rous sarcoma tissue after the 6-hour examination, and no intracellular virus was evident in the tissues 8 days after virus inoculation. All the cultures contained viable tissue throughout the course of the experiment, and Rous sarcoma virus was present in the tumor cultures.

To insure that Rous sarcoma virus was not interfering with the growth of influenza or psittacosis viruses in the eggs used for titration, Rous virus was injected into either the allantoic or the yolk sac before injection of influenza or psittacosis virus, respectively, and no effect was observed on the subsequent growth of these two viruses, thus excluding the possibility of a viral interference reaction.

Table III summarizes the results of the experimentation with the vaccinia virus. It is

TABLE III. Growth of Vaccinia Virus.

Tissue	Virus content: Inoculum	6 hr	2nd washing at 6 hr	2 days	4 days	6 days	8 days
Chick embryo	$10^{1.6}$*	F = $10^{4.47}$	$10^{2.91}$	F = $10^{3.12}$	F = $10^{5.5}$	F = $10^{5.16}$	—
	$10^{1.55}$	F = $10^{4.21}$	$10^{2.5}$	F = $10^{1.63}$	F = $10^{3.3}$	F = $10^{4.5}$	F = $10^{2.77}$ T = $10^{2.25}$
Adult chick	$10^{1.63}$	F = $10^{3.63}$	$10^{2.5}$	F = $10^{2.3}$	F = $10^{2.65}$	—	F = 10^{4} T = $10^{5.66}$
	$10^{5.6}$	F = $10^{6.25}$	$10^{3.5}$	F = $10^{3.26}$	F = $10^{1.32}$	F = $10^{5.56}$	—
Rous sarcoma	$10^{1.55}$	F = $10^{3.43}$	$10^{2.75}$	F = $10^{2.63}$	F = $10^{2.0}$	F = $10^{1.55}$	F = 0 T = 10^{1}
	$10^{5.6}$	F = 10^{5}	10^{1}	F = $10^{2.76}$	F = $10^{3.4}$	F = $10^{2.61}$	—
Virus controls	$10^{1.55}$	F = $10^{3.5}$	—	F = 10^{3}	F = $10^{2.8}$	F = $10^{1.76}$	0
Tissue "	0	0	—	0	—	0	—

* LD_{50}.

F = fluids, T = tissues, — = not tested.

evident that the concentration of the virus in the medium of the embryo cultures rose slowly but progressively from that present in the cultures when the unadsorbed virus was removed. Though the multiplication of the vaccinia virus was slow, it did increase in titer, while the virus in control cultures was found to lose its infectivity slowly and to disappear from the culture medium in 6 to 8 days.

In other studies using similar technics, growth curves similar to this presented for vaccinia virus in embryo skin-muscle tissue have been found in cultures of chick embryo heart, brain, gut and liver tissues(12).

The course of infection of the adult chick fibroblastic tissue by the vaccine virus was also indicative of low-grade virus multiplication. Fluids removed from the cultures at 2-day intervals demonstrated progressively increasing amounts of the virus, regardless of concentration of the original inoculum. Results similar to these have also been found in vaccinia virus infections of adult chick brain, heart, gut and liver tissues under identical *in vitro* conditions (unpublished observations).

The results with the vaccinia virus in the tumor tissue were not as clearly definable as those above. In 2 separate experiments, the concentration of the virus recovered from the cultures after 2 washings with BSS 6 hours after virus inoculation was approximately 70 to 90 times less than the inoculum. At 2-day intervals thereafter, the concentration of the virus demonstrable in the culture fluids, completely removed for each titration, was progressively less than that obtained at the previous examination with one exception. By the 8th postinoculation day, there was no active virus in the fluids and only a small amount present in the tissues.

If one considers only that this growth pattern shows a progressive diminution in titer, comparing favorably to that of the control cultures, there is no evidence of vaccinia virus multiplication. The results could then be explained on the basis of elution and/or dilution of the virus in the cultures with continual thermal inactivation. But if one considers that the virus in the control flasks was not removed from the culture at 2, 4 or 6 days, while the fluids of the experimental tissue flasks were totally removed and titered at 48-hour intervals, with unattached virus being washed out before replenishment of the medium, it seems more likely that these results indicate a low-grade reproduction of vaccinia virus in the Rous tissue. Definitive interpretation awaits more detailed experiments, but it is probable that vaccinia virus is able to proliferate slowly in Rous sarcoma cells in which Rous sarcoma virus was demonstrable.

The results of the growth of Newcastle Disease virus in the cultures of the various tissues are summarized in Table IV and indicate multiplication of NDV in all 3 tissues, but viral reproduction was much slower in adult

TABLE IV. Growth of NDV Virus.

Tissue	Virus content: Inoculum	6 hr	2nd washing at 6 hr	2 days	4 days	6 days	8 days
Chick embryo	$10^{8.5}$*	$10^{2.75}$	$10^{1.5}$	$10^{8.57}$	$10^{6.58}$	$10^{6.26}$	—
Adult chick	$10^{8.71}$	—	$10^{1.69}$	F = 10^{3} T = $10^{1.87}$	F = 10^{4} T = $10^{3.56}$	F = $10^{4.61}$ T = $10^{3.4}$	F = 10^{4} T = $10^{4.64}$
Rous sarcoma	$10^{8.5}$	F = $10^{2.4}$ T = 10^{2}	10^{1}	F = $10^{1.43}$ T = 10^{1}	F = $10^{3.5}$ T = $10^{4.24}$	F = $10^{3.73}$ T = $10^{2.5}$	F = 10^{4} T = 10^{5}
	$10^{5.5}$	F = 10^{3}	10^{2}	F = $10^{1.63}$	F = 10^{2}	F = $10^{4.26}$	F = 10^{2} $10^{6.2}$
Virus control	$10^{8.71}$	$10^{1.87}$	—	0	0	0	0
	$10^{5.5}$	$10^{3.23}$	—	0	0	0	0
Tissue "	0	0	—	0	—	0	—

* LD_{50}.

F = fluids, T = tissues, — = not tested.

chick and Rous sarcoma tissues than in embryo tissues. The Rous sarcoma tissues were shown to contain the causative virus.

Discussion. The results of the experiments in which embryo and normal adult chick tissue cultures were inoculated with 4 animal viruses illustrates a definite qualitative difference in susceptibility to virus infection by closely related tissues, since neither NWS nor psittacosis viruses were able to propagate in the adult tissue while multiplying freely in the embryo fibroblastic cells. NDV and vaccinia virus, on the other hand, were capable of proliferation in both types of tissues.

Such a difference in susceptibility of embryonic and adult chick tissues to infection by an A strain of influenza virus was also found by Colville and co-workers(12) while studying the factors important in the *in vitro* propagation of the PR-8 virus in chick tissues. The fact that the NWS strain of influenza A virus, which seems to multiply in a larger variety of tissues than any other influenza A virus, gives results comparable to those found by Colville *et al.* suggests that there is some uniform insusceptibility of adult chick fibroblastic cells to the A strains of influenza viruses.

That psittacosis virus should prove incapable of reproduction in adult fibroblastic tissue was surprising in light of its capacity to grow in a variety of chick embryo tissues *in vitro* and the native parasitism of closely related viruses in the chicken *in vivo*. Regarding the latter, however, although the virus has been shown to infect chickens both experimentally and naturally, no report has been made of its recovery from blood-free skin-muscle tissues. This appears to be the first recorded attempt to cultivate the virus in chick fibroblastic tissue *in vitro*. Nevertheless, these experiments illustrate 2 interesting examples of an absolute difference in the susceptibility of embryo and chick tissues to virus infections *in vitro*.

In spite of the fact that all 4 viruses studied have previously been shown to multiply in malignant tissue(1) and some in virus-induced tumors, only NDV and vaccinia virus were capable of reproduction in the Rous sarcoma cultures. This represents a dual infection of the Rous cell by both the causative Rous agent and the invading NDV or vaccinia virus.

The finding that influenza and psittacosis viruses are incapable of multiplying in Rous sarcoma tissues *in vitro* is compatible with two possible explanations. First, one might attribute this failure of viral growth to a viral interference reaction exerted by the Rous sarcoma virus already present in the cells. However, the results obtained with the adult chick tissues present a second possibility, for in cultures of these 2 related types of tissue only those viruses capable of replication in the adult connective tissue and muscle elements were similarly capable of growing in the sarcomatous tissue derived from the adult fibroblastic tissue. The results obtained from the Rous sarcoma cultures indicate either that the Rous cells are themselves insusceptible to

all of the 4 agents studied and the NDV and vaccinia virus infect only normal chick cells present as "contaminants" in the cultures of the malignant tissue, or that in the transformation to malignancy the Rous cells retain certain biological properties of the normal adult chick cell, manifest here in susceptibility to virus infection, and in this respect are more like adult than embryonic tissue.

Of these possibilities, the latter seems more feasible. It is entirely reasonable to assume that certain biological elements of the normal adult chick cell are retained even after the cell is transformed into a malignant state by the Rous virus and that these properties may be intimately related to the intracellular mechanisms necessary for virus reproduction. Although it cannot be stated with certainty that the Rous cultures were "pure" in that no nonmalignant chick cells were present, it can be reasonably assumed that the few such cells present could not serve adequately as host tissue for the large quantities of NDV produced. However, with vaccinia virus the low titers obtained in Rous tissue prevent exclusion for this same reason.

It appears to the authors, therefore, that under the conditions of the present studies the ability of an extraneous virus to infect the Rous sarcoma cells is dependent on certain biological properties retained by the cell from its nonmalignant predecessor and is independent of the presence and reproduction of the causative Rous agent already inhabiting the cell. If this is true, the Rous cell system differs from that reported by Ginder and Friedewald(13) in their experiments with rabbit fibroma tissues because these workers described the growth of Semliki Forest virus in the rabbit fibroma tissue both *in vivo* and *in vitro*, but the virus failed to proliferate in normal rabbit connective tissue elements.

Summary. The ability of 4 animal viruses (NDV, NWS, vaccinia and psittacosis) to propagate in Maitland-type tissue cultures of chick embryo and adult chick fibroblastic tissues and Rous sarcoma tissue was studied and all 4 viruses were found capable of proliferation in the embryo tissue, while only NDV and vaccinia virus multiplied in the adult chick and Rous sarcoma tissue cultures. Thus, Rous sarcoma tissue appears to be more closely related to connective tissue of adult chicks than to that of chick embryos in its susceptibility to infection with certain other viruses. NDV and Rous sarcoma virus can produce a dual infection of cells.

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