ARTHROPOD TRANSMISSION OF RABBIT FIBROMATOSIS (SHOPE)

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(With Plates 1–2)

INTRODUCTION

The concern over human cancer makes it desirable to study neoplasms of lower mammals, with the hope that information concerning the mechanisms of their initiation and proliferation may be applicable to the human disease. One such neoplastic disease is Shope's fibroma of rabbits (Shope, 1932) which occurs naturally in cottontails (*Sylvilagus floridanus* subsp.) in several localities of eastern and midwestern United States. These virus-induced fibromas normally regress after several weeks of cellular reproduction and thereby do not satisfy one of the requirements of a virus as a causative agent of cancer, namely, that it stimulate the host cell to continuous aggresssive proliferation (Pinkerton, 1952). However, if small amounts of virus are injected into the skin of new-born domestic rabbits, the resulting tumours will grow progressively, inducing satellite growths, sometimes generalized fibromatosis, and often will kill the host (Duran-Reynals, 1945). Dalmat (1957) also reported the histological picture of a 203-day cottontail fibroma suggestive of malignant transformation.

A few investigators (Philip, 1942; Kilham & Woke, 1953; Kilham & Dalmat, 1955; Dalmat, 1957, 1958*a*, *b*) have shown that suitable arthropods transmit fibromatosis as has also been demonstrated for myxomatosis of rabbits (Aragão, 1943; Fenner, Day & Woodroofe, 1952).

The present paper is a report of several studies attempting to elucidate the cycle of infection and the mode of transmission of the rabbit fibroma. Kilham & Dalmat (1955) found that arthropods could transmit fibroma virus from cottontail rabbits to other cottontails or to domestic rabbits, but they were unable to transmit to either host from fibromas of domestic rabbits, although the virus titre of the latter was equal to that of the cottontail fibromas. For this reason, cottontails were used as the donor hosts in the experiments herein reported, except where specific attempts were made to transmit from the domestic rabbit tumours.

MATERIALS AND METHODS

Virus

Except where otherwise indicated the virus used was the Boerlage strain, originally isolated by Shope (Fenner & Woodroofe, 1954) in 1947 from a New Jersey

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cottontail. The virus was passed intradermally in domestic and cottontail rabbits. Portions of excised tumours were stored in vials with beef infusion broth at −20° C. The stock virus preparation was a 10% suspension made by adding beef infusion broth to either stored or fresh tumour tissue that had been ground in a mortar with the aid of sterile Alundum and cleared by centrifugation at 650 *g* for 7 min. Tenfold dilutions were prepared as needed and these were inoculated intradermally into test animals in 0·25 ml. doses.

Animals

Cottontail rabbits (*Sylvilagus floridanus* subsp.) were supplied by Mr Paul Bruce Dowling, Resident Biologist of the Busch Memorial Wildlife Area, Missouri Conservation Commission and by Dr Carleton M. Herman, Chief of the Wildlife Pathology Section, Patuxent Research Refuge of the U.S. Fish and Wildlife Service, Laurel, Maryland. The domestic test rabbits (*Oryctolagus cuniculus*) were usually of the New Zealand strain and weighed 1800–2000 g.

Treatment of the rabbits with the carcinogens, methylcholanthrene and 1, 2, 5, 6-dibenzanthracene, and with X-rays has already been described (Dalmat, 1958*a*).

The mice employed in brain passage studies were 1-day-old National Institutes of Health (NIH) general purpose mice. The techniques used have been described previously (Dalmat, 1958*b*).

Arthropods

The arthropods used for transmission were the mosquitoes, *Aedes aegypti* (L.), *A. triseriatus* (Say), *Culex quinquefasciatus* Say, and *Anopheles quadrimaculatus* Say; the reduviid bugs were *Rhodnius prolixus* Stål, *Triatoma infestans* Klug, and *Triatoma phyllosoma pallidipennis* (Stål); the bedbug, *Cimex lectularius* L.; and the chigger mite, *Trombicula splendens* Ewing. These were all from laboratory colonies, housed in an insectary maintained at approximately 28° C. and 50–70% relative humidity. Mosquitoes to be fed on tumours were usually 2–5 days old and had not had a previous blood meal. Just prior to feeding they were transferred to a glass cylinder, 1½ by 3 in., both ends of which were covered with cotton netting through which the mosquitoes probed. After feeding, the mosquitoes were transferred to glass lantern chimneys that fitted snugly into the covers of Petri dishes previously lined with moistened cellucotton and two layers of filter-paper. The top of the chimney was covered with cotton netting. The bugs were kept in 250 ml. gauze-covered beakers with pieces of paper towelling within for supports. After feeding, the engorged bugs were transferred to clean beakers.

No convenient holder was found for maintaining chiggers on the tumours for the 3 days required for engorgement. The experimental method used has been given in the body of the paper.

Except where otherwise indicated, stock suspensions of any of the arthropod species were prepared from separate pools of twenty heads, thoraces and abdomens. These pools were first ground in 1 ml. lots of beef infusion broth, then centrifuged at 650 *g* for 7 min. and the respective supernatants taken as 10% stock suspensions

from which tenfold dilutions were prepared. The standard quantity of inoculum used was 0·25 ml.

Inoculations of mosquitoes with various suspensions were effected by an adaptation (Dalmat, 1957) of Weathersby's (1952) technique, using capillary pipettes inserted into rubber tubing that was connected to the laboratory vacuum system.

The dilution technique involving the feeding of mosquito tissue to other mosquitoes was that of Merrill & TenBroeck (1934).

EXPERIMENTAL

Mechanism of fibroma transmission

Arthropods capable of transmitting fibroma

Reduviid bugs. Experiments were conducted to determine whether the reduviids (conenosed bugs) could transmit only by interrupted meals as found by Philip (1942), or whether virus is able to survive in their bodies and be transmitted some time later to recipient hosts. The results are tabulated in Table 1. It can be

Table 1. *Transmission of fibroma virus by reduviids**

	Donor rabbit			Interval from original infective meal to transfer (days)	Reaction of rabbit to		
						Inoculation of	
Expt.	Type	Age of tumour (days)	Mode of passage†		Bites	Head pools	Abdomen pools
A	Cottontail	27	D.F., H.S., A.S.	14	+‡	+ +	−
B	Cottontail	92	I.M., H.S., A.S.	0	About 50 tumours coalesced	+ + + + +	+ +
C	Cottontail	92	D.F.	22	Several + + + tumours	.	.
D	Cottontail	135	D.F., H.S., A.S.	7	+ + + + + coalesced	+ + + +	+ +
E§	Domestic	12	I.M., H.S., A.S.	0	—	−	−
F§	Domestic	12	D.F.	10	—	.	.
G§	Domestic	12	D.F., H.S., A.S.	25	—	+	+ +
H§	Domestic	37	D.F., H.S., A.S.	7	Several + + + and + + + + tumours	+ + + +	+ +
I§	Domestic	19	I.M., H.S., A.S.	0	−	−	−
J§	Domestic	19	D.F., H.S., A.S.	21	Several + areas	+ +	+

* *Rhodnius prolixus* was used in all experiments except A, in which *Triatoma infectans* and *T. phylloma pallidipennis* were also used. All passages were made to albino domestic rabbits.

† D.F. = delayed feeding; H.S. = suspension of mosquito heads; A.S. = suspension of mosquito abdonens; I.M. = interrupted meal. All tests performed with group of ten bugs.

‡ Approximate size of fibroma tumour; each + equals 0·25 in. in diameter.

§ The tumours of the donor rabbit used in these experiments were the result of arthropod transmission.

seen that in Expt. A, *Triatoma infestans, T. phyllosoma pallidipennis* and *Rhodnius prolixus* transmitted the virus by feeding 14 days after their initial infective meal on cottontail fibromas. Inoculation of their mouthparts also gave positive results, but no virus seemed to be present in their abdomens. The relatively light infection in this experiment was probably due to the particular strain of virus used in

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initiating the tumours in the donor cottontail. This material was not of the Boerlage strain, but derived from tumours of an experimentally infected western cottontail which had shown delayed development and delayed infectivity for arthropods. Since *R. prolixus* was the species of reduviid most abundant, it was used in all subsequent studies with Boerlage virus. As shown in Table 1, it was able to transmit virus from both cottontails and domestic tumours, both by interrupted meals (Pl. 1, fig. 1) and after delays up to 25 days. Inoculation of mouthparts of those bugs that had just re-fed also resulted in positive tumour formation, as did inoculation of suspensions of abdomens. Suspensions of thoraces were not used, since this body region always had given negative results upon inoculation.

It is interesting to note that in Expts. E and I, in which there was no interval between the original infective meal of *Rhodnius* and the attempted transmission to a recipient rabbit, all results were negative; however, in Expts. G and J, in which 25 and 21 days, respectively, elapsed between the infective meal and transmission trials, the results were positive. This may be construed as an indication of possible virus proliferation in the bugs, but the variations encountered in various groups of experiments makes it difficult to accept such an interpretation.

Under normal circumstances mosquitoes usually are not able to transmit virus from one domestic rabbit to another. However, when the tumours of domestic donor rabbits endure longer than the usual 10–12 days, mosquitoes often can pick up and transmit virus. It is noteworthy that tumours resulting from the bite of infected reduviids did seem to last longer than usual and were infective when fed on by other reduviids. The mechanism involved has not yet been determined.

Bedbugs. In one study eighty bedbugs were fed on a 30- to 50-day-old naturally occurring fibroma of a Maryland cottontail. About forty of these were given only a partial meal and were then permitted to continue feeding to engorgement on a clean domestic rabbit. Inoculations were also made of suspensions of the heads of both groups at dilutions 10^{-1} to 10^{-4}, and of the abdomens at 10^{-1} only. Those bedbugs that fed to repletion on the cottontail tumours were re-fed 7 days later on another domestic rabbit. Very large tumours (Pl. 1, fig. 2) were formed as a result of interrupted feeding of the bedbugs; inoculation of mouthpart dilutions resulted in a titre of at least 10^{-3} (greatest dilution prepared), and the inoculum of the abdomen suspension also produced a large tumour mass equivalent in size to that produced by the 10^{-1} dilution of the mouthpart suspension. Feeding delayed for 7 days after the initial infective meal resulted in a similarly large tumour mass, and the inoculation of suspensions of the mouthparts and abdomens of these bugs resulted in significant tumours, although the titre of the mouthparts was lower than that of bugs tested after an interrupted meal.

In another study, bedbugs were permitted to feed on tumours of domestic rabbits that had been infected by bedbug bites. Transmission was accomplished only by inoculation of head suspensions of the bugs, but not by their interrupted meal. The tumours produced in the recipient rabbits were rather small. In general, it was found that the virus titres of tumours produced by interrupted feeding of bedbugs were much higher than those of tumours produced as a result of delayed feeding.

Chigger mites. Early in the course of the experimental work, it was felt that

chiggers might be involved in the natural transmission of fibroma virus. Certainly, reduviids and bedbugs had not been reported from cottontail rabbits in nature, and fleas were shown to be poor vectors (Kilham & Woke, 1953). It was reasoned that the virus had to pass the cold months of the year either in cottontail rabbits or in the arthropod vector. Infected rabbits trapped during December in upper New York State and during January in Missouri, had tumours corresponding to experimental lesions of 1 month's duration. This indicated that the virus probably survived the winter in the mammalian host, but it evoked speculation on the vector involved. Mosquitoes probably would not be active in New York during November or in Missouri during December. It was therefore reasoned that chiggers might well be important vectors, since the virus might pass the winter in their eggs. The report by Ryckman & Roos (1955) of a mouse, *Perognathus pernix pernix*, bearing five squamous papillomata in the rump region that were heavily parasitized with chiggers further supported the possibility of chigger involvement in the natural history of cottontail fibroma.

Experiments were conducted to determine the capacity of one species of chigger, *Trombicula splendens*, to transmit fibroma virus. Since only the larval form attacks mammals, transmission would have to be transovarial, infection in rabbits taking place by feeding of newly hatched larvae that emerged from eggs of adult chiggers, which, in turn, had developed from an earlier generation of larvae which had fed on fibromas. The complete cycle, from larvae feeding on fibromas to feeding of a subsequent generation of larvae on a recipient rabbit, involved at least $2\frac{1}{2}$ months.

The difficulty encountered in maintaining larval chiggers on tumours limited the number of attempts to accomplish experimental transmission. With the technique finally adopted, larvae were placed individually on the tumours along with a drop of water with a fine camel's hair brush. By the time the drop evaporated, the chiggers usually had attached. The engorged chigger larvae, which usually left the host in 3–4 days, were collected from the water-detergent trap over which the infested rabbits were kept. They were maintained in Mason jars coated on the inside with activated charcoal and plaster of Paris, and with about $\frac{1}{2}$ in. of vermiculite on the bottom. In each of three experiments, approximately 100 larvae were placed on the tumour. In one run fifty engorged larvae were recovered while in the other two runs thirty-five and nineteen were recovered. In each case, 100 of the resulting larvae of the subsequent generation were fed on a recipient rabbit and an equal number were macerated and suspended for inoculation into the same animal. In no case did tumours develop.

Mosquitoes. In preliminary studies (Kilham & Dalmat, 1955) it was shown that *Anopheles quadrimaculatus*, *Culex pipiens*, and *Aedes triseriatus* would transmit fibroma virus to domestic rabbits 8 days after these mosquitoes had full blood meals on cottontail fibromas. *A. aegypti* was able to transmit up to 5 weeks after the infective meal.

Throughout the course of the present work, the ability of mosquitoes to transmit fibromas was borne out. All species used, *A. aegypti*, *A. triseriatus*, *Culex pipiens*, *C. quinquefasciatus*, and *Anopheles quadrimaculatus* were efficient vectors.

Because of the ease of handling and rearing, *Aedes aegypti* was chosen as the standard for most of the experimental work.

Transmission potential of arthropods

It is well known that when a group of individual arthropods is exposed to a disease, every individual of the group does not become infected and not every one that does become infected will transmit the infection with each subsequent meal. Since groups of arthropods, rather than individual ones, were used for feeding studies and pools of mouthparts or abdomens were used as inocula, it became necessary to know what proportion of the population of an arthropod species did become infected with the virus and what size pool of body parts was necessary in order to assure constant results that would permit comparisons between different experiments. In a group of six experiments designed to determine whether or not infected mosquitoes gradually lost their virus with repeated meals, individual mosquitoes were permitted to probe serially on a recipient rabbit. It was found that tumour production was irregular and that there was no definite relation between the number of previous probes and the size of the tumours. Many probes produced no tumours while subsequent probes did produce tumours, often larger than those resulting from the initial probes.

In each of two experiments, 100 *A. aegypti* were fed on an infective cottontail tumour. Seven days later, each of the mosquitoes was fed individually on a marked area of a recipient rabbit host. The bites of 73 % of the mosquitoes in one experiment and of 64 % in the other resulted in tumours. In another experiment, 200 mosquitoes were fed on an infective tumour of a cottontail rabbit. On the following day, three groups of suspensions containing 1, 2, 10 or 20 ground mosquito heads were prepared, each group in 0·5 ml. of beef infusion broth. These were considered as 10^{-1} suspensions and from them 10^{-2} to 10^{-5} dilutions were prepared. In each of three test rabbits four series of dilutions prepared from the 1, 5, 10 and 20 heads were inoculated. In one of the test rabbits the suspension prepared with 1 or 5 heads both titred 10^{-3}, while the suspensions prepared with 10 or 20 heads both titred 10^{-4}. However, in the two other recipient rabbits, the titration of the suspension prepared with only 1 head resulted negatively, while the suspension of 5 heads again titred one log less than those with 10 or 20 heads. In another experiment, suspensions of 2 heads and 5 heads both gave erratic results.

In similar studies with *Rhodnius prolixus*, it was found that eight out of ten bugs transmitted virus as demonstrated by tumour formation. With these, suspensions of 5, 10, or 20 heads resulted in constant titres of 10^{-3}.

It was decided that in all experiments involving mosquitoes, groups of twenty would be used in order to increase the probability of positive transfer by innoculation of heads of mosquitoes that had fed on low titre tumours, and also to strengthen the chance of obtaining tumour formation by re-feeding of such mosquitoes.

Effect of blood meals on arthropod transmission

Several experiments were set up to determine the possible importance of previous blood meals of the arthropods in evaluating transmission studies. Half of the

mosquitoes involved had been given a blood meal 1 week previous to the infectious meal, while the other half, which had emerged on the same day, had been fed on a sucrose solution only. Both groups of mosquitoes were permitted to feed on a cottontail fibroma known to be infective. Three days later they were re-fed on a fresh domestic rabbit and suspensions of the mouthparts of each group were also titrated in the same recipient host. No difference was found as a result of the re-feeding of the two groups of mosquitoes. However, for the sake of uniformity, mosquitoes 2–5 days old, that had not had a previous blood meal, were used. Due to the relatively long life cycle of the reduviids and bedbugs, the nymphs had to be used as well as the adults. Because of the need for blood meals for nymphal development, almost all individuals of these groups of insects would have had blood meals prior to their infective meal.

Localization of fibroma virus in arthropods

Before determining whether or not fibroma virus proliferates in the arthropod vector, it was necessary to know exactly where in the arthropod the virus lodged. Fenner *et al.* (1952) found that mosquitoes transmitted the related myxoma virus mechanically on their mouthparts and they likened them to 'flying pins'. These investigators were able to transmit from primary skin lesions using steel pins as mechanical analogies of mosquitoes. Kilham & Woke (1953) succeeded in inducing fibromas in cottontails by sticking them with pins that had just been inserted into fibromas of an infected cottontail. To call the mosquito a 'flying pin' would indicate that the virus adhered to the outside of their mouthparts as it would have to in the case of a pin. To test this the present investigator examined more minutely pin transmission of fibromas and the location of the virus in the infected mosquitoes.

Transmission by pins. As a control 100 *A. aegypti* were fed on a cottontail fibroma with a virus titre of 10^{-6}, twenty of these being permitted to take only a partial blood meal and then to continue their meal on a normal domestic rabbit. Fifty no. 5 japanned insect pins, inserted in corks, were individually stuck into the same cottontail fibroma to a depth of about 4 mm. After approximately 10 sec. they were removed and forty of the pins were arranged in lantern chimneys in the same manner as mosquitoes. Ten of the pins were immediately used to prick the skin of the normal domestic rabbit and all sites were marked. Within 6 days small tumours started developing at the sites where pins had been inserted into the test animal, while within 3 days tumours were apparent at the sites of interrupted mosquito feeding.

On each of the 4 days after pricking of the cottontail tumour, ten of the pins which had been kept in lantern chimneys in the insectary were inserted into the skin of a healthy second test rabbit; on the second to fourth day after their full blood meal, twenty of the mosquitoes that had fed on the tumour were permitted to re-feed on this same test rabbit. No tumours developed where pins had been used, but they did form as a result of the re-feeding of the mosquitoes.

In another experiment four pins were used, each of these being inserted into the healthy skin of a test domestic rabbit in five marked areas within ½ hr. of pricking

the cottontail tumour. The first two of the five areas serially pricked by two of the pins developed tumours, while only the first sites penetrated by the other two pins had tumours develop. It became obvious that, although mosquitoes appeared to serve as pins when transmitting virus by interrupted feeding, they maintained their ability to transmit for a long period of time, while pins could not transmit virus after the day of contamination. This indicated that the virus probably did not cling only to the exterior of the mosquito mouthparts.

In experiments in which the mouthparts of large numbers of *A. aegypti* were brushed with a concentrate of high titre virus suspension, Kilham & Dalmat (1955) found that transmission by the bite of the treated mosquitoes was unsuccessful. Although inoculation of a mouthpart suspension of some of these mosquitoes, prepared immediately after they were bathed with the virus, did result in low grade tumours, similar preparations on the following 3 days were completely negative.

It appeared from these studies that only virus that lodges between the stylets or within the head capsule will survive and be transmitted.

Biting through virus-moistened skin. To substantiate further the findings that mosquitoes do not serve only as 'flying pins' in the transmission of fibroma virus as they have been found to do in myxoma studies (Fenner *et al.* 1952; Day, 1955), a distinct type of experiment was carried out. The flanks of two domestic rabbits and two cottontails were shaved and moistened in marked areas with fibroma virus (titre 10^{-6}). One hundred *A. aegypti* were permitted to feed through the virus-moistened skin of each rabbit and then twenty of the dissected mosquito heads of each group were prepared in a 10% suspension that was inoculated into another site on the same rabbit upon which the mosquitoes had fed. Four days later the remaining mosquitoes of each group were permitted to feed on a marked area of a fresh domestic rabbit and the mouthparts of twenty of each group were prepared in suspensions that were inoculated into marked sites on the same test animal. All areas on which mosquitoes had fed or into which the mouthpart suspensions had been inoculated remained negative. Challenge proved the rabbits to be susceptible.

In a repetition of the above experiments both *A. aegypti* and *Rhodnius prolixus* were used. Neither species caused tumours to develop by feeding through the virus-moistened skin, nor did tumours develop by inoculation of mouthpart suspensions. However, re-feeding of forty remaining mosquitoes on the healthy skin of another test rabbit 3 days later resulted in a single small tumour; a small tumour also developed in an area of a test rabbit where the twenty remaining *Rhodnius* were permitted to re-feed 8 days after feeding through the virus-moistened skin. These results conclusively show that the mosquito mouthparts do not serve merely as 'flying pins'. The few positive areas on test rabbits probably resulted from virus particles that had been taken up into the reduviid and mosquito mouthparts while previously feeding through the virus-moistened skin. These results were similar to those found by Rendtorff & Wilcox (1957) when they attempted to introduce fibroma virus by the penetration of larvae of *Nippostrongylus muris* through the skin of rabbits. There can be little doubt that in the case of

arthropods, virus must actually enter between the stylets of the mouthparts to effectuate subsequent transmission.

In experiments in which mosquitoes were permitted to feed on cotton pledgets soaked with virus suspension of cottontail tumours with high titres, the mosquitoes never became infectious. Titrations of the suspension after it was associated with the pledgets for a few days showed that it did not appreciably lose its virulence. In similar studies with myxoma, Day, Fenner, Woodroofe & McIntyre (1956) interpreted their negative results as probably due to a decrease of virus titre in such preparations to below the threshold necessary for mosquito transmission.

From the negative results in the studies on the ability of mosquitoes to transmit after the exterior of their mouthparts had been contaminated with virus, or after biting through virus-moistened skin or cotton, it appears that the virus can enter the mouthparts only while the mosquitoes actually probe a tumour. Probably the mosquitoes are unable to free virus from cells loosely held together in tissue suspensions. This belief was supported in a preliminary study in which mosquitoes were shown capable of transmitting virus from a cell-free virus preparation poured over cotton pledgets.

Virus in arthropod faeces. In myxomatosis, it has been shown that virus imbibed in blood by mosquitoes is excreted and plays no part in the transmission of the disease; only infected skin lesions are a source of virus capable of rendering mosquitoes infectious (Fenner *et al.* 1952; Day *et al.* 1956). To determine whether or not fibroma virus is also excreted in the faeces, 200 *Aedes aegypti* were fed on an infective tumour and were thereafter transferred in equal numbers to two lantern-chimney holding cages. One of the chimneys was so arranged with a piece of screening that the mosquitoes were unable to make contact with the moist filter-paper beneath. After 2 days, the filter-papers were removed and separately placed in phosphate buffer (pH 6·8) overnight to remove as much virus as possible and the liquid of each was then passed through filter-paper and centrifuged at 3200 *g* for 20 min. to clear the suspension. The supernatant fluid was then spun in a Model 'L' Spinco preparative ultracentrifuge at 35,000 *g* for 1 hr. to produce a virus pellet which was taken up in about 10 ml. of buffer and beef infusion broth in equal volume. This was inoculated into four rabbits. The suspension prepared from the faeces of mosquitoes that passed through the screening produced no tumours, while that prepared from the paper not screened from the mosquitoes did result in tumour formation. It seemed likely that virus found on the unscreened paper resulted from the probes of mosquitoes attempting to imbibe water rather than from the faeces that had been deposited on it.

It is difficult to understand why all myxoma virus imbibed with blood during meals taken at sites other than skin lesions should pass through the alimentary tract of the mosquitoes (Day *et al.* 1956); it would seem that some virus particles, whether from the lesions or blood, would adhere to the mouthparts. Possibly the relatively low concentration of virus particles in the blood accounts for the inability of mosquitoes to transmit from sites other than primary lesions.

On the other hand, although there is no apparent viraemia in fibroma infections (Kilham & Fisher, 1954), it would be expected that sufficient virus would be

picked up by mosquitoes feeding on the skin lesions so that a portion might pass through the alimentary tract with the blood meal and be recovered in the faeces. This could not be shown in attempts at isolation from papers contaminated with faeces. Virus was never recovered from pools of mosquito thoraces and only eighteen isolations have been made from several hundred abdominal pools.

Attempts to isolate virus from various parts of arthropods. Two hundred *A. aegypti* were fed on a cottontail fibroma known to be infective for mosquitoes and, at varying intervals thereafter, aliquots of twenty mosquitoes each were dissected and separate pools made of the stylets, palpi, clypeal region, head capsule, thorax, legs, and abdomen. The pools were prepared in suspensions with beef infusion broth and 0·25 ml. of each was inoculated into the shaved flank of a normal domestic rabbit. The results are shown in Table 2.

Table 2. *Localization of fibroma virus in* Aedes aegypti *by inoculation into rabbits*

Aliquots*	Interval from infectious meal to inoculation (days)	Mosquito body parts inoculated						
		Stylets	Clypeus	Palpi	Head capsule	Thorax	Legs	Abdomen
A	0	+ + + +†	+ + +	−	−	−	−	−
B	2	+ + +	+ + +	−	−	−	−	−
C	2	+ + +	+ + +	−	−	−	−	+
D	3	+ + + + +	+ + +	−	+ + +	−	−	−
E	6	+ + + + +	+ + + +	−	+	−	−	−
F	8	+ + + + +	+ + + + +	−	+	−	−	−

* Twenty mosquitoes per aliquot.

† Relative development of fibromas; each + equals 0·25 ml. in diameter.

It can be seen that the stylets served as the principal reservoir for virus particles, with the clypeal region almost as important. The other body parts did not seem to play an important part in the storage of virus. Table 2 also suggests an increase in the size of tumours produced by inoculation of the stylets and clypeus with increased intervals between the infective meal and dissection. This might have been due to an increase in the number of virus particles, to the lack of homogeneity amongst the members of the mosquito pool, to variation in reaction of different host animals to the inocula, or to some slight variation in technique. In further experiments along the same line, pools of body parts from different aliquots were prepared in suspensions, stored at $-20°$ C. until all dissections had been made, and then all pools were inoculated into the flanks of two rabbits. The tumour size was somewhat greater as a result of suspensions prepared at longer intervals from the infective meal of the mosquitoes, but not as marked as in the series shown in Table 2. In these tests also the stylet and clypeus pools always resulted in tumour formation, the abdomen pools rarely did, while the head capsule pools showed the presence of virus irregularly. Day *et al.* (1956) found the virus concentration of myxoma greater in the head than in the mouthparts on the day of the infectious meal, but the present investigator never found this to be the case with fibroma.

Day *et al.* (1956), in myxoma studies patterned after those of Kilham and Dalmat (1955) for the purpose of comparison, fed *A. aegypti* on myxoma tumours and then dissected the mosquitoes at intervals after their infective meal, inoculating the pooled body parts into test rabbits. In spite of the fact that these mosquitoes picked up virus primarily from skin lesions rather than from the blood stream, the investigators still recovered small amounts of virus from the thoraces and considerable virus from the abdomens. The abdominal concentration fell within a few days after the infective meal. These findings with myxoma virus in mosquitoes do not parallel the findings with fibroma virus. The high concentration of virus in the abdomens of mosquitoes soon after their infective meal on myxoma lesions, and the absence of virus in the abdomens of mosquitoes that fed on fibromas, probably reflect the high viraemia associated with myxomatosis and the absence of demonstrable viraemia in fibroma infections.

In experiments using reduviids and bedbugs for transmission of fibroma, the bugs were not dissected beyond the three principal body regions. The heads of both groups always contained virus while the thoraces never did. With the reduviids, the abdomens appeared to contain virus more regularly several days to weeks after the infective meal than they did the day of the meal. In the two experiments using bedbugs, some virus was found in the abdomens, both on the day of the infective meal and 7 days later. The finding of fibroma virus in the abdomen of reduviids after long periods from the infective meal appears contrary to what is found with myxoma in mosquitoes (Day *et al.* 1956).

Day *et al.* (1956) present a photomicrograph of the maxillae of *A. aegypti* showing a row of recurved teeth toward the apex. They believe that the maxillae of the mosquito offer the greatest opportunity for lodgement of virus particles and with subsequent probes the virus particles would be wiped off onto the new host. Light and electron microscope preparations have supported their contention. However, the inner surface of the labrum-epipharynx also has numerous tooth-like projections which also may harbour virus particles. While feeding, it is conceivable that the virus is dislodged into a viscous liquid that bathes all the stylets in the labium and it may be carried by diffusion of the fluid with saliva to the wound formed by the action of the maxillae. Very little is actually known of the cellular structure of the mouthparts.

There was no experimental evidence that fibroma virus could be passed transovarially in mosquitoes. Inoculation of the eggs of infected females did not produce fibromas on test rabbits. Adults developing from larvae kept in a suspension of virus or inoculated with the suspension also did not produce lesions, nor did inoculation of the washed larvae.

Multiplication of fibroma virus in arthropods

It is generally accepted that in biological transmission by arthropods, the pathogen passes through the tissues of the vector; in mechanical transmission the pathogen is carried purely mechanically by the vector. According to Day (1955) all virus–vector relations so far described are covered by these categories and no virus has been shown to be transmitted by more than one mechanism. In most of

the arthropod-borne diseases there is biological transmission but with the insect-borne pox viruses of animals, transmission appears to be mechanical. The work of Brody (1936) on fowlpox, of Shope (1940) on swinepox, Day & Fenner (1953) on mousepox, and of Philip (1942) on rabbit fibroma suggested that transmission was mechanical.

As evidence that myxoma is mechanically transmitted to rabbits by arthropods, Day *et al.* (1956) presented the following: Virus is acquired from skin lesions and not from the blood. Virus in the mosquito midgut does not induce infection. Virus injected into the haemocoele does not multiply. There is no latent period between the acquisition of virus and the ability to transmit, and interrupted feeding usually causes infection. Virus concentration in the mosquito decreases with time. The probability that mosquitoes will cause infection decreases with each probe. There is no vector specificity.

In laboratory studies of *Anopheles atroparvus* in relation to myxomatosis in England, Andrewes, Muirhead-Thomson & Stevenson (1956) found evidence for the possibility of virus multiplication. Semi-hibernating mosquitoes retained their infectivity under conditions simulating those of winter shelters up to 220 days. Even during the summer months, virus retained its titre in infected insects for several weeks. However, virus survived on the mouthparts of dead mosquitoes for only a few days. These investigators obtained results with myxoma similar to those shown by Kilham & Dalmat (1955) with fibroma, but they were not able to demonstrate actual multiplication of the virus. Jacotot, Toumanoff, Vallée & Virat (1954) believed that multiplication of myxoma virus took place in *A. atroparvus* on the evidence that transmission occurred up to 21 days after the infective blood meal; that a single bite was sufficient to produce infection, and that a single mosquito could infect several rabbits one after the other at short intervals. These authors also were unable to show actual multiplication of the virus in the mosquitoes.

Since fibroma virus is antigenically and morphologically related to myxoma virus and is also considered to be of the pox group, several types of experiments were performed to determine whether fibroma was transmitted mechanically or whether there might be biological transmission. Virus proliferation in the mosquito was considered the main criterion for biological transmission.

Periodic titration of suspensions of infected mosquitoes

Thirty-five series of experiments, similar to those of Whitman with yellow fever (1937) were run with fibroma virus, in each of which a single arthropod species was fed on fibromas known to be infective. At intervals thereafter, aliquots of the particular arthropod were dissected into their head, thorax, and abdominal region and tenfold dilutions of each were inoculated into a test rabbit to determine the virus titre (Pl. 1, fig 3). In all but seven series mosquitoes were used. Approximately 200 were fed on the tumour of known titre, and usually twenty of these were dissected at each titration, the first group on the day of the infectious meal and the remaining groups at varying intervals. With reduviids and bedbugs the techniques were the same except that ten insects were pooled instead of twenty. If

the virus titre fell soon after the infective meal and then subsequently rose again, virus multiplication was considered a possibility.

In about half of the experiments there were indications of virus proliferation. In Table 3 the results of one experiment using *Aedes aegypti* are given. In this series, head pools only were used and only fifteen heads were included in a pool. It can be seen that the first two suspensions prepared on the day of the infective meal and on day 3, as well as that prepared 17 days after feeding, titred 10^{-4}, while the intervening titrations never exceeded 10^{-3}. Likewise, as a result of periodic inoculations of the head pools, the number of days required for maximum tumour development increased after the day of infection and then gradually decreased. The size of tumours produced by inocula of a particular dilution differed somewhat according to the interval from the infective meal to the time of inoculation, but in general it did not appear to be noticeably smaller as this interval increased. These data indicate the possibility of virus proliferation.

The results of periodic titrations of the head pools of *Anopheles quadrimaculatus* that had previously fed on infectious fibromas are presented in Table 4. There appears to be a definite decline in titre soon after the infective meal and then a gradual return to the original level. Again, the time required to reach the maximum titre in each experiment increased after the infective meal and then dropped. It should not be expected that the virus titre would be greater at the end of the experiment; growth requirements of the virus might surpass the capacity of mosquito cells to supply them and virus present in mosquitoes would be limited thereby.

Transmission by reduviids was already discussed in the previous section under 'Arthropods capable of transmitting fibroma'. From Table 1 it can be seen that the virus remained virulent in the bugs as long as 25 days and that, in some experiments, the mouthparts were negative on inoculation the day of the infective feed but positive a few weeks later.

As previously stated, only half of the numerous experiments performed with periodic titrations gave indications of possible multiplication but, in general, it was found that virus could at least persist in the arthropod mouthparts for as long as 35 days and could be transmitted even after seven intervening blood meals between the infective and the final feedings.

Effect of successive re-feeding of mosquitoes on concentration of virus

A large number of mosquitoes was fed on an infectious cottontail tumour and a pool of mouthparts of twenty of them was titrated in a test rabbit. At intervals thereafter the remaining mosquitoes were re-fed on a susceptible test rabbit and, after each re-feeding, twenty were dissected and a suspension of mouthparts titrated in the same rabbit.

In one experiment, re-feedings were accomplished 3, 7, 9 and 12 days after the infective meal. Titrations were read 10 days after the inoculation of the dilutions into the test rabbits, and the results of the bites were read at the same time. Only mosquitoes that actually had fed on the uninfected test rabbits were kept for subsequent re-feeding so that all aliquots would be homogeneous as far as the number of blood meals taken was concerned.

Table 3. *Periodic titrations of suspensions of head pools* of* Aedes aegypti *infected with fibroma virus†*

Days from infective meal to inoculation	Days from inoculation to examination	Development of tumours resulting from head pool inocula‡							
		10^{-1}		10^{-2}		10^{-3}		10^{-4}	
		Right	Left	Right	Left	Right	Left	Right	Left
0	5	+	+	+	+	±	+	−	−
	10	+ +	+ +	+ +	+ + +	+ +	+ +	+	+
	13§	+ +	+ +	+ + +	+ + + + ‖	+ +	+ + +	+	+
	17	+ +	+	+ +	+ + + ‖	+ +	+ + +	+	±
	23	+ +	+	+ +	+ + + ‖	+	+ + +	−	−
	30	+ +	−	+ +	+ + + ‖	+	+ + +	−	−
3	2	−	0¶	−	−	−	−	−	−
	7	+ + +	0	+ +	+ +	+	+ +	−	−
	10	+ + +	0	+ +	+ + +	+	+ +	±	+
	14	+ + + +	0	+ +	+ + +	+	+ +	+	+
	18	+ + + +	0	+ + +	+ + +	+ +	+ +	+	+
	21§	+ + + +	0	+ + + +	+ + + + +	+ + +	+ + +	+	+
	28	+ + + +	0	+ + + +	+ + + + +	+ + + ‖	+ + + ‖	+	+
5	5	−	0	−	−	−	−	−	−
	8	+	0	+	+	−	+	−	−
	12	+ + +	0	+ +	+ +	−	+ +	−	−
	16§	+ + +	0	+ +	+ + +	+	+ +	−	−
	19	+ + + +	0	+ +	+ + +	+	+ +	−	−
	26	+ + + +	0	+ +	+ + + ‖	−	+ +	−	−
7	3	+	0	−	−	−	−	−	−
	6	±	0	−	±	−	−	−	−
	10	+ + +	0	+ +	+ + +	+ +	−	−	−
	14§	+ + + +	0	+ +	+ + + +	+ +	−	−	−
	17	+ + + +	0	+ +	+ + + +	+ +	−	−	−
	24	+ + + + +	0	+ +	+ + +	+ +	−	−	−
10	3	−	0	−	−	−	−	−	−
	7	+	0	−	+	−	+	−	±
	11	+ + + +	0	−	+ +	−	+ +	−	±
	14§	+ + + + +	0	−	+ + +	−	+ + +	−	−
	21	+ + + + +	−	−	+ + +	−	+ + +	−	−
12	5	−	0	−	−	−	−	−	−
	9	+ + +	0	+ +	+ +	+	±	−	−
	12§	+ + +	0	+ +	+ +	+	+	−	−
	19	+ + +	0	+ +	+ +	±	±	−	−
17	4	−	0	−	−	−	−	−	−
	7	+ +	0	+ +	+ + +	−	+ +	+	+
	14§	+ + + +	0	+ + +	+ + +	+	+ +	+	+

* Each pool composed of fifteen mosquitoes.

† All mosquitoes had fed on a naturally occurring cottontail fibroma, and titrations were made in domestic rabbits.

‡ Each + represents 0·25 in. in diameter of the tumour; ± indicates a reaction less than + (not considered positive).

‖ Crusting and regressing.

§ Number of days for maximum development of tumours.

¶ No inoculation made in sites with 0.

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Table 5 indicates that after four re-feedings the virus titre of the head pool was approximately the same as after the original infective meal. Also, with each re-feeding, the number of tumours produced per mosquito feeding was not reduced,

Table 4. *Periodic titrations of suspensions of head pools* of* Anopheles quadrimaculatus *infected with fibroma virus*†

Expt.	Interval from infective meal to inoculation of mouthparts (days)	Development of tumours‡ resulting from head pool inocula				Time to reach maximum titre (days)
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	
A	0	+ + + +	+ + + +	+ + +	−	14
	6	+ + +	−	−	−	15
	9	+ + + +	+ + + +	+	−	12
	11	+ + + + +	+ + + + +	+ + +	+	10
B	0	+ + +	+ +	+	+	10
	2	+ + +	+	−	−	14
	7	+ + +	+	+	−	13
	11	+ + + ½	+ + + ½	+	−	10
	15	+ + +	+ +	+	±	10

* Each pool composed of twenty mosquito heads.

† All mosquitoes had fed on naturally occurring cottontail fibromas and titrations were done in domestic rabbits.

‡ Titration is expressed as dilutions of mouthpart suspension that gave positive tumour development. Each + represents 0·25 in. of diameter of tumour; ± indicates a reaction less than + (not considered positive).

Table 5. *Effect of periodic re-feeding of* Aedes aegypti* *on titre of fibroma virus*

Days from infective meal to refeeding and/or titration	Results of titrations					Results of re-feedings	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	No. mosquitoes feeding	No. tumours produced
0	+ + + ½†	+ + +	+ +	+	−	Initial infectious meal	
3	+ +	+ +	+	−	−	120	12, + + to + + ½
7	+ + +	+ + +	+	+	−	74	5, each + + +
9	+ + +	+ + +	+	−	−	48	6, + + to + + +
12	+ + +	+ + +	+	−	−	25	6, each + +

* Each pool for titration in domestic rabbit prepared from twenty mosquitoes.

† Each + represents 0·25 in. of diameter of tumour.

but rather increased. It is difficult to be certain whether each tumour was the result of the bite of a single mosquito or whether several tumours might have resulted from successive probes of one mosquito. The results, however, again would indicate possible virus proliferation. In several experiments in which the mosquitoes had become weakened, they did not feed with normal vigour and the resultant tumours were often fewer and smaller.

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Effect of successive probes of mosquitoes on concentration of fibroma virus

It was thought that if transmission of fibroma virus were purely mechanical, successive probes of an infected mosquito should result in the wiping off of all virus particles, and finally no tumours should result.

A small group of *Aedes aegypti* was fed on a fibroma of a cottontail rabbit known to be infective for mosquitoes. Two days later individuals of the group were induced to probe successively on marked areas on the back of a normal domestic

Table 6. *Effect of successive probes of* Aedes aegypti *on presence of fibroma virus on their mouthparts*

Mosquito	Mosquito meal	Result of probes*
1	Original	+ +½ + + − − − + ++ − − − − +
	Repetition after 2 days	+ − ++ − − − − + ++ − − − +
2	Original	+++ +++ − ++ ++ + − + − − − − +½ ++ ++
	Repetition after 2 days	++ + − − − +++ − − + − ++ −
3	Original	++ ++ + ++ ++ ++ − + + ++ ++ ++ ++ ++
	Repetition after 2 days	− + + +½ +½
4	Original	+ + + + − + − − − − − −
	Repetition after 2 days	− + + +½ − +½

* Read left to right for first to last probes of series. Each + represents 0·25 in. of diameter of tumour.

rabbit with the intent of 'wiping off' virus adhering to the mouthparts. Usually one mosquito would make twelve to fourteen probes. After an interval of 1–2 days, the same mosquitoes were induced to repeat this activity. Typical results are found in Table 6. Since the probes followed successively, it would be expected that in one series a point close to virus extinction might be reached. If after an intervening period a second series of probes again were to give rise to tumours, virus multiplication might well be suspected. The sequence of tumours resulting from the successive probes of mosquitoes 1, 2 and 3 were more typical than of mosquito 4. With the first two mosquitoes, both the original series of probes and the second series after a 2-day interval resulted in sporadic and apparently haphazard tumour formation. Both series of probes of mosquito 3 quite consistently resulted in tumours, and there was no indication of a definite drop in virus concentration. It cannot be stated that the virus was completely wiped off since, after many negative probes, tumours arose. Conversely, there was no enhancement of tumour growth after the 2-day interval. However, it is quite obvious that the virus was present in sufficient concentration to induce tumours with serial probes and that tumour size did not gradually decrease.

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Inoculation of fibroma virus into haemocoele of mosquitoes

If a known quantity of fibroma virus could be inoculated into mosquitoes and aliquots then be titrated in a test rabbit at various intervals after they had been inoculated, a quantitative measure of proliferation could be had. Should it be found that the virus inoculated into the haemocoele did multiply, it would lend added weight to the possibility of biological transmission. A similar procedure was employed by Hurlbut (1951) relative to the relation of Japanese B encephalitis to mosquitoes. He serially inoculated ground-infected mosquito material into other mosquitoes and thereby proved virus proliferation.

Out of 100 *A. aegypti* in Expt. A inoculated with approximately 0·002 ml. of a virus suspension titring 10^{-7}, 85 % survived. After feeding on a normal domestic rabbit, forty were dissected and separate suspensions were prepared of pooled mouthparts, head, thoraces, legs, and abdomens, and after dilution (10^{-4}) were inoculated into the flanks of the same test rabbit. Five days after the haemocoele inoculations, the remaining forty mosquitoes were permitted to feed on a second domestic rabbit and were then similarly dissected, the suspensions being inoculated into a single rabbit. In Expt. B the same procedures were followed but feedings and titrations were carried out 1, 5 and 7 days after the haemocoeles had been injected with virus. The results of these experiments are given in Table 7.

With parenteral inoculation, the virus was recovered from all body regions, except the mouthparts, up to the second day in Expt. A and as late as the fifth day in Expt. B, but by the fifth and seventh days, respectively, all attempts at virus recovery failed. The tumours formed in Expt. B were small and multiple, suggesting a non-homogenous inoculum. However, when this rabbit tissue was prepared in suspension and inoculated into another rabbit intradermally, similar tumour clusters developed. Several other experiments corroborated the findings of Expts. A and B, the virus usually disappearing after 2 days. Day *et al.* (1956) who experienced a complete disappearance of myxoma virus in similar experiments, did not mention from which regions virus was recovered.

Clearly, virus never reached the mouthparts of the inoculated mosquitoes since no transmission occurred by bite. It is difficult to understand why virus that reached the head capsule of mosquitoes feeding on infectious tumours was still localized in the head and initiated tumours upon inoculation 8 days after the infectious meal (Table 3), while that which reached the head capsule or legs during the process of injecting virus into the body cavity seemed to disappear usually within 2 days (Table 7). To date, nothing is known of the mechanism which brings about the disappearance of virus from the head, thorax, and abdomen of the inoculated mosquitoes. What is needed now is a systematic examination of possible sites of virus elimination and/or destruction in the mosquito (e.g. gut, haemolymph factors other than blood cells, fat or protein globules, epithelial cells).

Passage of fibroma virus by serially feeding mosquitoes to mosquitoes

Since periodic titration of infected mosquitoes in itself may not always be a convincing method of demonstrating virus multiplication, serial feeding of

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mosquitoes to other mosquitoes was made starting with a group known to lodge fibroma virus. If, after several dilutions by this technique, the recipient mosquitoes still give a titre equivalent to that of the initial group, there could be little doubt that multiplication occurred.

Table 7. *Fate of fibroma virus after inoculation into haemocoele of* Aedes aegypti

Expt.	Interval from inoculation to titration (days)	Body region inoculated	Results of titrations* of mosquito body parts in domestic rabbits 10^{-1}	10^{-2}	10^{-3}	10^{-4}	Results of feeding
A	2	Mouthparts	−	−	−	−	−
		Heads	+ + + +	+	−	−	−
		Thorax	+ + +	±	−	−	−
		Abdomen	+ + +	±	−	−	−
		Legs	+ +	−	−	−	−
	5	Mouthparts	Completely negative				
		Heads					
		Thorax					
		Abdomen					
		Legs					
B	1	Mouthparts	−	−	−	−	−
		Head	+ + + + †	+	−	−	−
		Thorax	+ + + + †	+	−	−	−
		Abdomen	+ + + + †	±	−	−	−
		Legs	+ + + †	−	−	−	−
	5	Mouthparts	−	−	−	−	−
		Head	+ + + †	±	−	−	−
		Thorax	+ + + †	−	−	−	−
		Abdomen	+ + + †	−	−	−	−
		Legs	+ †	−	−	−	−
	7	Mouthparts	Completely negative				
		Head					
		Thorax					
		Abdomen					
		Legs					

* Each + represents 0·25 in. in diameter of tumour growth; ± indicates a reaction less than + (not considered positive). Each pool composed of forty mosquitoes.

† These tumours were composed of small discrete units.

Five experiments were performed, using the technique of Merrill & TenBroeck (1934) who successfully demonstrated ten serial passages of equine encephalomyelitis virus in *A. aegypti*. The body parts of mosquitoes that had fed on infective tumours were ground and prepared in suspensions that were fed to newly emerged mosquitoes as well as titrated in rabbits. This procedure was continued through five passages. The original group of mosquitoes that had fed on cottontail fibromas was shown to be infective when part of the suspension prepared from their heads was titrated in domestic rabbits. The suspensions of tumours on which the mosquitoes fed in each experiment had titres of 10^{-6} or 10^{-7} and the suspensions of mosquitoes prepared from those that had fed on the tumours had titres of

10^{-5} or 10^{-6}. Virus was never recovered from mosquitoes that had fed on suspensions of ground mosquitoes, or even from those that fed on the suspension of known infected mosquitoes.

Stability of fibroma virus

Although definite proliferation of virus in the mosquitoes has not been shown, virus was able to persist for long periods, even when the mosquitoes had taken several blood meals between their infective meal and their final meal or inoculation into a test rabbit. Is stability of the virus correlated with the observed longevity?

In one experiment the mouthparts of *A. aegypti* that previously had fed on infectious fibroma tumours were ground and prepared in a 10% suspension with beef infusion broth which was then diluted in tenfold increments to 10^{-5}. The titre in a domestic rabbit was 10^{-4}. These dilutions were then left at room temperature (23° C.) in cotton-stoppered test-tubes for 18 days. At the end of this period the preparations still titred 10^{-4} when inoculated into another rabbit. In another test with virus associated with mosquito mouthparts, the titre of the virus was reduced only one log after 35 days at room temperature.

Andrewes *et al.* (1956) found that myxoma virus survived only a short while in dead mosquito heads. This was considered as another indication of the need for the living mosquito in survival of the virus when outside of the rabbit host. However, the early disappearance of the virus could be accounted for by the absence of a suitable moist culture medium like the one used for preparing dilutions.

Seven tests to determine virus stability were carried out with tumour suspensions that were kept at room temperature for 33, 46, 75 and 92 days, respectively. All of them remained virulent, the three kept 92 days losing 1–2 logs and those kept 75 days losing 1 log only. The suspensions kept for 33 and 46 days gave titres as high at the end of the period as when first prepared. Thus, fibroma virus is very stable, and this may account, in part, for the prolonged infectivity of mosquitoes.

Relationships of fibromas to host and vector

Published reports of studies on fibroma virus are often concerned with its transmissibility from rabbit to rabbit by inoculation via intradermal, subcutaneous, intramuscular, or intratesticular routes (Shope, 1932; Ahlström & Andrewes, 1938). It has been common experience that tumour tissue of domestic or cottontail rabbits will infect the other species as well as that from which it is derived. Much time has also been given to studies of the immunological properties of the virus, its relation to myxoma virus and to others of the pox group, the effect of various chemical and physical agents on the development of the tumours (Hyde, 1936; Ahlström & Andrewes, 1938; Duran-Reynals, 1945; Harel & Constantin, 1954; Kilham & Fisher, 1954), and to the structure of the virus (Lloyd & Kahler, 1955).

Soon after his original isolation of the fibroma virus from a wild cottontail rabbit, Shope (1932) showed that tumours developing in domestic rabbits reached maximum size in about 10 days and then began to regress. In the natural cottontail host the tumours took 39 days to reach the peak titre and thereafter they

began to crust and regress. Kilham & Fisher (1954) found that the cottontail tumours reached their peak of development in 4–6 weeks and maintained their high titre for a variable period of time before regressing. In histological studies, they found that after the virus titre reaches its peak, there is little variation in the epidermal changes or configuration and the size of the fibroma cell inclusions. Virus titre of epidermis was always at least as high as that of underlying layers.

In studying the histology of fibromas taken from cottontail and domestic rabbits, Shope (1932) observed that the overlying epidermis of the cottontail fibroma exhibited a peculiar type of hyperplasia and degeneration reminiscent of molluscum contagiosum in man, a condition which was not found in the epidermis of domestic rabbit fibromas. He considered this to be a matter of species difference. Ahlström & Andrewes (1938) found that the reaction to fibroma virus in domestic rabbits treated with tar showed not only the same clinical course, but also the same histological type as the reaction in the wild cottontail rabbit.

The importance of histological differences between the normal host fibroma and that of the domestic rabbit became more evident during the course of investigations to determine the possible mode of transmission of this virus. This will be discussed fully by Dalmat & Stanton (in the press).

Duration of fibromas on rabbits

The duration of rabbit fibromas depends on age of the host, strain of rabbit, physical or chemical treatment of the rabbits, dose of virus employed, and origin or mode of inoculation of the virus.

Age of host. Domestic rabbit weanlings, weighing from 1800 to 2000 g. (about 8–9 weeks old), were inoculated with either standard suspensions of tumours with titres between 10^{-5} and 10^{-8}, or with tenfold dilutions of these suspensions. The resultant tumours were usually evident in about 3–5 days, reached their maximum size in 10–12 days, and thereafter crusted and regressed until they had completely disappeared by 16–20 days. This clinical picture can be varied by intradermal inoculations of virus suspensions of other than tumour-mash origin, and by using methods of inoculation other than the hypodermic syringe. Out of several hundred tumours of weanling domestic rabbits, only about twenty-seven remained intact more than 16 days. Of these, one endured for 105 days, while all the others regressed by 39 days. All but one of the twenty-seven had been induced by the feeding of infected mosquitoes or inoculation of head pools of infected mosquitoes or reduviid bugs, or had developed in a domestic rabbit strain other than the common albino stock. The use of X-rays, 1,2,5,6-dibenzanthracene, and methylcholanthrene (Dalmat, 1958*a*), or passage of the virus through the mouse brain (Dalmat, 1958*b*), also caused domestic rabbit tumours to persist for longer periods than usual.

In suckling domestic rabbits inoculated with 0·1 ml. doses of tumour-mash suspension of weanling domestic rabbits or of cottontails, tumours were apparent at 4–5 days. They followed closely the development of fibromas in weanling rabbits up to 10 days, but then continued to grow up to 19 days (Pl. 1, fig. 4), with damaged areas commonly being regenerated. If the suckling was inoculated with suspensions of suckling tumour origin, the new tumours became apparent on the

second day, and in 6 days they surpassed the size normally reached in a weanling rabbit in 10 days. These tumours continued to grow, becoming very high and widespread by 13 days. The titre of suspensions of some of these tumours surpassed 10^{-8}. Many of the inoculated sucklings died of the infection within 6–8 days, and almost all were dead by 21 days.

In the cottontail, the age of the rabbit is qualitatively of prime importance to the duration of tumours. Animals known to be well over a year old were quite refractory to infection and their tumours regressed within a month. Early regression also was encountered in some of the younger cottontails, but their tumours usually continued developing for 3–5 months. Tumours produced by inocula of greater dilution or by mosquito transmission appear to persist better than those of suspensions of greater concentration or those prepared from tumour mash. In general, the cottontail tumours first became apparent in 7–8 days, reached maximum size in about 3 weeks, and then persisted for about 2–3 months before crusting and showing definite signs of regression. The peak titre usually was 10^{-6}. In areas that were scratched or in other ways injured, crust formation and a certain amount of regeneration usually occurred up to 4 months after infection.

Relation of extractable virus in tumours to the titre of the inoculum initiating them

Very little work has been published on factors responsible for limiting the size of tumours or on the mechanism of regression. A plausible explanation is that the size of the tumour is related to the number of rabbit cells initially infected. Since the time of virus-burst should be constant for all dilutions in a titration series, sites in which a larger number of cells are initially infected will have a geometrically larger number of cells attacked when cell division occurs, and hence larger resultant tumours. When sufficient antibody is built up in the rabbit to prevent further cell infection, all tumour sites are affected at the same time and regression sets in. The mechanism by which antibody inhibits further development of the intracellular virus is not known.

In studies on Rous sarcoma virus, Bryan, Calnan & Moloney (1955) found a definite relation between the amount of virus extractable from experimentally produced tumours and the amount of virus contained in the inoculum that had been used for inducing them. The extractable virus (per unit of weight of tumour) was found to approach a maximal concentration in tumours induced by relatively strong doses, but to be correlated highly with the dose at lower initiating dose levels. Weak inocula containing only one 50% effective unit or less produced tumours from which relatively little or no virus could be recovered on extraction. These investigators concluded that the amount of extractable virus was related primarily to the initiating dose, rather than secondarily through the influence of the time of development and growth, which is also related to dose. Therefore, the general assumption that the absence of demonstrable virus in tumour-tissue extracts is justification for considering tumours to be of 'non-viral' origin, was shown to be invalid.

Is the factor limiting the development of fibromas similar to that in Rous sarcoma? A dilution series of standard tumour suspension was prepared in tenfold

steps and inoculated into a domestic rabbit. After 10 days each of the resultant tumours was harvested, weighed, minced, and ground separately, and 10% suspensions were prepared. A dilution series of the suspensions of each of the tumours was then inoculated into three test animals. The results of one such experiment, in which the titre of the original standard tumour suspension was 10^{-4}, are given in Pl. 2, fig. 5.

If the size or development of Shope's fibroma were to be controlled by a similar mechanism as that described by Bryan *et al.* (1955), then the titre of suspension A, prepared from the tumour initiated by the 10^{-1} dilution of the standard tissue suspension, should have been greater than the titre of suspensions B, C and D, which were prepared from tumours induced by the respective 10^{-2}, 10^{-3} and 10^{-4} dilutions of the same standard tissue suspension. However, it can be seen that this was not the case. The titres of suspensions A–D were approximately the same in spite of the dilution of their initiating inocula. As long as the particular virus strain had a titre of 10^{-4}, tumours initiated by whatever dilution of it still maintained approximately the same titre so long as the suspensions were prepared on a weight-volume basis. Thus, the amount of extractable virus per unit mass of fibroma tumour, unlike the situation with Rous sarcoma, is not related primarily to the initiating dose, but rather to strain virulence.

Infectivity of fibromas for arthropods

Cottontail rabbits. A. aegypti were fed at various intervals at the site where cottontail rabbits had received intradermal inoculations of fibroma virus. Grouping together all the animals used, the intervals were from 1–203 days. The mosquitoes were given either an interrupted meal, terminating it on a clean domestic rabbit, or at intervals after the full blood meal on the cottontail they were re-fed on a domestic rabbit and/or suspensions of their mouthparts were inoculated into a domestic rabbit. The results are incorporated in Table 8. In one instance transmission was accomplished by permitting mosquitoes, that had fed on a 7-day cottontail tumour 3 days previously, to re-feed on a domestic rabbit. The same tumour also was infective when used for transmission at 11, 18 and 25 days, after which it commenced to crust and regress. The donor cottontail had been inoculated with a suspension of testicular tissue. Whether the source of the inoculum had anything to do with the early infectivity of the resultant tumours for arthropods was not tested. However, the suspension used had a titre of 10^{-5}, certainly no higher than most suspensions used for infecting rabbits in other experiments. Table 8 indicates the earliest age at which the tumour of a particular rabbit was found infective for *A. aegypti*. In twenty-five tests, while mosquitoes were not able to transmit virus from cottontail tumours under 7 days of age, they always transmitted from tumours over 35 days old. Once the tumour became infective, it remained so until it was so crusted that mosquitoes could no longer feed through it. In general, the older the tumour the greater the likelihood that it would be infective.

Reduviid bugs were not fed systematically on cottontail tumours of different ages, but tumours of 17, 27, 92 and 135 days all proved infective for them. Bed-

bugs were able to transmit from naturally occurring tumours estimated to be 30–50 days old.

Domestic rabbits. The infectivity of domestic rabbit tumours was tested in a manner similar to that used for determining infectivity of cottontail rabbits. Of

Table 8. *Onset of infectivity of cottontail fibromas for* Aedes aegypti*

Age of tumours (days)	Number of rabbits proven infective†	Mode of transmission: Delayed re-feeding	Mode of transmission: Inoculation of mouthparts
7	1	1	−‡
10	4	3	4
11	1	1	−‡
15	1	−‡	1
17	1	1	−‡
19	2	−‡	2
20	1	1	1
21	1	1	1
22	1	−‡	1
24	1	−‡	1
26	1	1	1
27	1	1	1
28	1	1	1
35 or older	10	10	10

* In each trial a pool of twenty *A. aegypti* were either re-fed or dissected for pooling of mouthparts.

† This represents the number of rabbits whose tumours first showed infectivity for *A. aegypti* at the age given in the first column. The tumours of each rabbit remained infective for all subsequent transmission trials.

‡ Not attempted.

Table 9. *Onset of infectivity of domestic rabbit fibromas for* Aedes aegypti

Age of tumours (days)*	Source of inoculum	Mode of transmission†: Delayed re-feeding	Mode of transmission†: Inoculation of mouthparts
8	22-day Chinchilla rabbit tumour	−	+
12	Infected reduviid heads	+	+
14‡	Domestic tumour produced by mosquito bites	−	+
19‡	Infected reduviid heads	+	+
22	Feeding of mosquitoes	+	+
25	Infected mosquito heads	+	+
27‡	Feeding of mosquitoes	+	+
39	Infected mosquito heads	+	+
56–105 Old Chinchilla rabbit	Testicular tissue of domestic rabbit	+	+

* Tumours were all on albino domestic rabbits unless otherwise indicated. Each entry represents a single rabbit.

† The + indicates positive transmission. The − indicates that transmission was not attempted by the particular method.

‡ Also shown to be infective at some subsequent trials. Tumours of rabbit not so indicated either were not used for further attempts at transmission or gave negative results.

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several hundred domestic rabbits tested, the tumours of only twenty-seven remained intact more than 16 days, the others usually regressing by 12–14 days. Only ten rabbits bore fibromas that were infective to mosquitoes. The findings are summarized in Table 9. It will be noted that all domestic rabbits whose tumours became infective for mosquitoes were either of a strain different from the usual albino stock, or they had received inocula other than suspensions of domestic tumour serially passed by inoculation. Once these tumours became infective they did not, like cottontail tumours, retain their infectivity until regression took place; even those tumours that were found to be infective at some trials subsequent to the initial demonstration of their infectivity were not consistently so. Tumours derived from arthropod bites endured longer and were more likely to become infective to arthropods than those derived from tumour-mash inocula. Tumours induced in the Chinchilla strain of rabbit also seemed more likely to be infective to arthropods. This greater longevity was not accompanied by delayed development of the tumours nor of the virus titre. The maximum titre was usually reached by the tenth day and did not increase materially beyond that. The virus titre at the peak of development of domestic rabbit tumours was about equivalent to that of the cottontail tumours at their peak. However, the tumours of domestic rabbits at this stage normally were not infective to mosquitoes while those of cottontails were. Tumours, infective for arthropods, could be induced in domestic rabbits by special treatment of the host animals before infecting them (Dalmat, 1958*a*, *b*).

Suckling domestic rabbits. The infectivity of tumours of suckling domestic rabbits (inoculated when 7 or 9 days old) resembled that of the cottontails more than that of the weanling domestic rabbits. Tumours of sucklings that had been infected by suspensions of tumours of adult domestic rabbits were infective to mosquitoes when tested at 10, 12, 14, 16, 17, 20, 23, 25 and 32 days. Once a tumour was infective it usually remained so. Nine of fourteen such sucklings tested show infectivity, and those that were not infective were ones tested earlier than 10 days. Tumours of sucklings that had been inoculated with suckling tissue suspensions developed exceptionally large tumours irrespective of the concentration of the inoculum, and were infective for mosquitoes as early as 8 days.

In one study *A. aegypti* were fed on the 12-day tumours of a suckling and, on the same day and on the 4 following days, twenty of the mosquitoes were dissected and tenfold dilutions of the pooled heads with attached mouthparts were inoculated into a test weanling domestic rabbit (Table 10).

The data reveal that the virus titre in mosquitoes was highest immediately after the infective meal or 3 to 4 days subsequently.

DISCUSSION

This study has shown that reduviids, bedbugs, and mosquitoes are capable of transmitting fibroma virus. All three groups transmitted well by interrupted feeding, but only the reduviids and mosquitoes were efficient vectors to test animals after long intervals from their infectious meal on the tumours. Although trials with chiggers were unsuccessful, they do not definitely preclude the possi-

Table 10. *Successive titrations of suspensions of head pools* of* Aedes aegypti *infected by feeding on fibromas of a suckling domestic rabbit†*

Recipient rabbit	Interval from infective meal to inoculation of mouthparts (days)	Titration of mouthparts in domestic rabbits			
		10^{-1}	10^{-2}	10^{-3}	10^{-4}
91	0	+ + + +‡	+ + + +	+ + +	+ +
91*a*	1	+ + +	+ + +	−	−
91*b*	2	+ +	+	−	−
91*c*	3	+ + + +	+ + +	+ +	−
91*d*	4	+ + + +	+ + +	+ + +	±

* Each pool composed of twenty mosquitoes.

† The suckling rabbit tumours on which the mosquitoes fed was 12 days old.

‡ Each + represents 0·25 in. in diameter of the tumour produced; ± indicates a reaction less than + (not considered positive).

bility that chiggers do play a role in transmission of the virus. Owing to technical difficulties involved in handling the chiggers for such studies, it was difficult to know how many of the engorged larvae that were recovered actually fed on the tumours, rather than on uninfected sites; chiggers easily could have migrated to the base of the tumours where virus would not have been picked up. If the virus were to be passed through the egg, it would have to be determined whether all of the offspring of the chigger would be likely to become infected. It was noted that the chiggers attached themselves more readily to the cottontail rabbit than to the domestic; it may be that transmission from cottontail to cottontail, the sequence not attempted in this work, might have been more successful. Lastly, there may be some specificity of chiggers in so far as their ability to transmit; in these experiments only one species was used. Because of the extended time necessary for experiments with chiggers, and the problem of assuring their confinement on tumours, a sufficient number of trials with them should be carried out before they can be disregarded as possible vectors. Mice bearing papillomas heavily parasitized with chiggers (Ryckman & Roos, 1955) further indicate the possibility of their involvement in transmission of virus tumours of rabbits. It is known (Brennan & Wharton, 1950) that some species of chiggers attach themselves to their rodent hosts during the autumn and winter which corresponds to the time that transmission must have occurred in the case of naturally infected rabbits trapped in Missouri and New York State. During this same period mosquitoes would either have been in hibernation or in the egg stage. Although fleas would appear to be likely vectors, in limited experiments with three species found on rabbits (Kilham & Woke, 1953) re-feeding on a normal cottontail produced only flat, scabby thickenings rather than raised, circumscribed highly vascular lesions, and these took about $2\frac{1}{2}$ weeks to arise. Transmission did not occur when confined fleas were offered interrupted meals, but only when fleas, after feeding on a fibroma, were released into an appropriate cage. At best, the fleas did not appear to be efficient vectors.

Much of the evidence from fibroma-arthropod relations indicates that transmission of the virus is mechanical. Thus (1) it is acquired only from the skin lesions;

(2) that injected into the haemocoele does not develop but quickly disappears; (3) there is no latent period after the infectious meal, nor is there involvement of the salivary glands as in other arthropod-borne virus diseases in which there is 'biological' transmission; (4) the virus could not be passed serially from mosquito to mosquito; and (5) there appears to be no vector specificity. However, evidence given in this paper is against the 'flying pins' concept. Virus does not remain virulent on the outside of the mosquito mouthparts, but must be taken up between the stylets for transmission to occur; once the insect takes an infectious meal it retains its infectivity for rabbits for long periods of time (possibly for the life of the vector), despite periodic blood meals on uninfected hosts and probing into moist filter-paper or through cloth covering their feeding tubes. In experiments entailing the periodic titration of mouthparts of infected arthropods, it was frequently found that virus titre dropped soon after mosquitoes fed on tumours and subsequently rose again. This suggests virus proliferation. In favour of this is the inability to cause virus extinction by successive probes of infected mosquitoes into test rabbits.

It is not necessary to demonstrate an initial loss of infectivity followed by a rise of virus titre in order to consider the possibility of virus proliferation, for if the mouthparts furnish a favourable environment for the virus, a reduction or slight rise in titre may easily not be demonstrable; the ability of the virus to maintain itself, but not to proliferate, may indicate that the mouthparts are unable to support additional virus. Against this view is the extreme stability of the virus which hardly loses titre at room temperature either as a suspension of tumour tissue or of ground mosquito mouthparts.

In the studies on the infectivity of fibromas for arthropods it was shown that the tumours of domestic weanling rabbits generally were not infective, even when at their peak of titre, while those of domestic sucklings and wild cottontails were infective, although their virus titre did not exceed that of the tumours of domestic weanlings.

It is valuable to compare the difference in infectivity of fibromas for arthropods with the difference in infectivity of domestic rabbit papillomas for cottontails and domestic rabbits. The papilloma virus passes from infected to susceptible cottontails under laboratory and natural conditions and usually can be recovered readily from the growths it causes on their skin. It can also be passed to domestic rabbits with resultant warts, but in this case the virus usually cannot be recovered. However, there is convincing serological evidence that the virus is present in the warts of domestic rabbits since their sera, or that of other domestic animals that are inoculated with extracts of these papillomas, will show the development of a specific antibody to the virus. Some investigators (Shope, 1937; Kidd, 1939) believe that the papilloma virus is actually present in the recipient domestic rabbits in a 'masked' form. Day *et al.* (1956) state that fibroma virus infection of domestic rabbits by mosquitoes sometimes occurs without the development of a detectable tumour. This was demonstrated by positive neutralization tests using sera of rabbits that were bitten by mosquitoes that had previously fed on domestic rabbit fibromas (Day, 1957). Of course, fibromas can be transmitted from domestic to

domestic rabbit by inoculation of tissue suspensions; it is only when arthropods are used as vectors that domestic rabbit tumours appear to be non-infective.

Kilham & Dalmat (1955) considered the possibility of the existence of some type of 'maturation' of tumours usually not reached by domestic rabbit fibromas even at their peak of development, but attained by tumours of cottontails. This factor might control infectivity or lack of infectivity of tumours for arthropods. This hypothesis was borne out in experiments to show the relationship of the morphology of fibromas to their infectivity for arthropods (Dalmat & Stanton, in the press).

In considering transmission of fibromatosis in nature, Philip (1942) was of the opinion that the frequency of fibroma lesions on legs and haunches of naturally infected cottontails indicated that some biting inhabitant of the nest or warrens might be involved. Although he successfully achieved transmission from fibroma lesions by interrupted feeding of reduviid bugs, re-feedings at intervals after an infective meal were all negative. He felt that if direct feeding on active fibromatous lesions was a prerequisite to transfer, it was difficult to visualize the mechanism responsible for observed natural incidence of the disease. Shope (1949) mentioned that since fibromas are more restricted to the feet of rabbits in nature, it seemed to him that the virus was transmitted by some intermediate host that inhabits the soil, perhaps in woodchuck burrows that cottontails frequent, and gains access to the feet of the rabbits by penetration. He supposed that it could be some worm parasite of rabbits that penetrates the skin in a manner analogous to that of hookworms in man. In response to Philip's difficulty in correlating the need for arthropods to feed on the lesions themselves for successful transmission with the rate of natural infection, Australian investigators working on the transmission of immunologically related myxomatosis (Fenner *et al.* 1952; Day *et al.* 1956) demonstrated that mosquitoes were the principal vectors of the disease, although transmission was successful only after feeding on the primary myxoma lesions. The present investigator has also found that the mosquitoes are particularly attracted to the vascular tumours, since they usually will not feed on densely haired regions. In an endemic area in Maryland, tumours have been found naturally occurring on the nose and around the eyes of cottontails with as much frequency as on the femora or feet. Kilham & Dalmat (1955) found that free-flying infected mosquitoes transmitted lesions to the ears and nose as well as to the legs. Rendtorff & Wilcox (1957) showed that the application of larvae of the nematode *Nippostrongylus muris*, when mixed with a suspension of rabbit fibroma virus, did not produce tumours on rabbits.

Since it has been shown that several insects can transmit the disease, and that they can maintain the virus between the stylets of the mouthparts for long periods, it seems that arthropods still are the most likely vectors of fibromatosis in nature. Studies should be made in areas in which fibromatosis is endemic to determine the species of biting arthropods that feed on the cottontail rabbits as well as the parasites that attack them. Should an epizootic then occur, natural infection studies could be carried out and the actual vectors determined.

SUMMARY

The mosquitoes *Aedes aegypti*, *A. triseriatus*, *Culex pipiens*, *C. quinquefasciatus*, and *Anopheles quadrimaculatus* were all found to be efficient experimental vectors of Shope's virus-induced fibromas of cottontail rabbits, transmitting the virus during interrupted feedings as well as after long intervals from an infective meal. The reduviid bugs, *Triatoma infestans*, *T. phyllosoma pallidipennis* and *Rhodnius prolixus*, and the bedbug, *Cimex lectularius*, were also capable of transmitting fibroma by interrupted or delayed feeding.

Evidence from various types of experiments indicated that arthropod transmission is mechanical, the virus being extremely stable in the insects. Some experiments did indicate the possibility of virus proliferation. Although mosquitoes did seem to serve as 'flying pins' when transmitting virus by interrupted feeding, they certainly were distinctive in that they maintained their ability to transmit for very long periods of time. To transmit fibromas, arthropods actually must draw virus up between the stylets of the mouthparts; mosquitoes were unable to transmit by feeding through skin moistened with a suspension of fibroma virus or by feeding subsequent to having their mouthparts painted with a virus suspension.

While cottontail tumours at peak virus titres are always infective for suitable insects, the fibromas of adult domestic rabbits generally are not infective, even though the virus titre is equivalent. However, the tumours of suckling domestic rabbits do become infective for insects.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Nine-day fibromas resulting from interrupted feeding of *Rhodnius prolixus* after beginning its meal on a cottontail tumour.

Fig. 2. Thirteen-day fibromas produced by interrupted feeding of bedbugs that had just fed on a cottontail tumour (left) and by inoculation of 10^{-3} through 10^{-1} dilutions of a head suspension of these bugs and 10^{-1} dilution of an abdomen suspension (group at right reading left to right.)

Fig. 3. Eight-day fibromas resulting from inoculations of 10^{-1} through 10^{-5} dilutions (left to right) of mouthparts of infected mosquitoes in typical titration.

Fig. 4. Seventeen-day fibromas of sucking domestic rabbit induced by intracutaneous inoculation when the animal was 9 days old.

PLATE 2

Fig. 5. Twelve-day fibromas resulting from titrations of similarly prepared suspensions of four fibromas that had been induced by tenfold dilutions of a single standard tumour suspension. A–D: titrations of tumours that had been induced by 10^{-1} to 10^{-4} dilutions, respectively.

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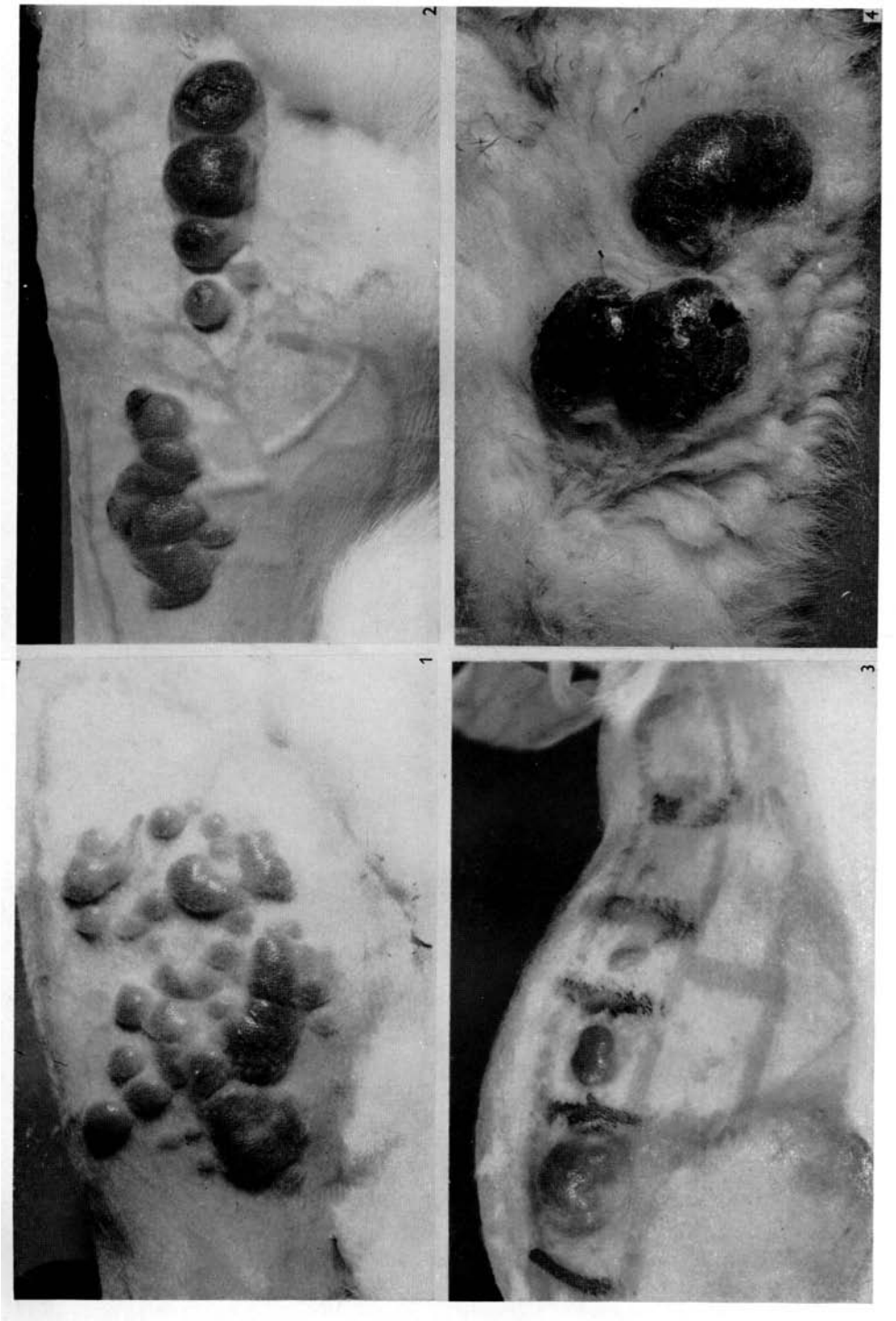

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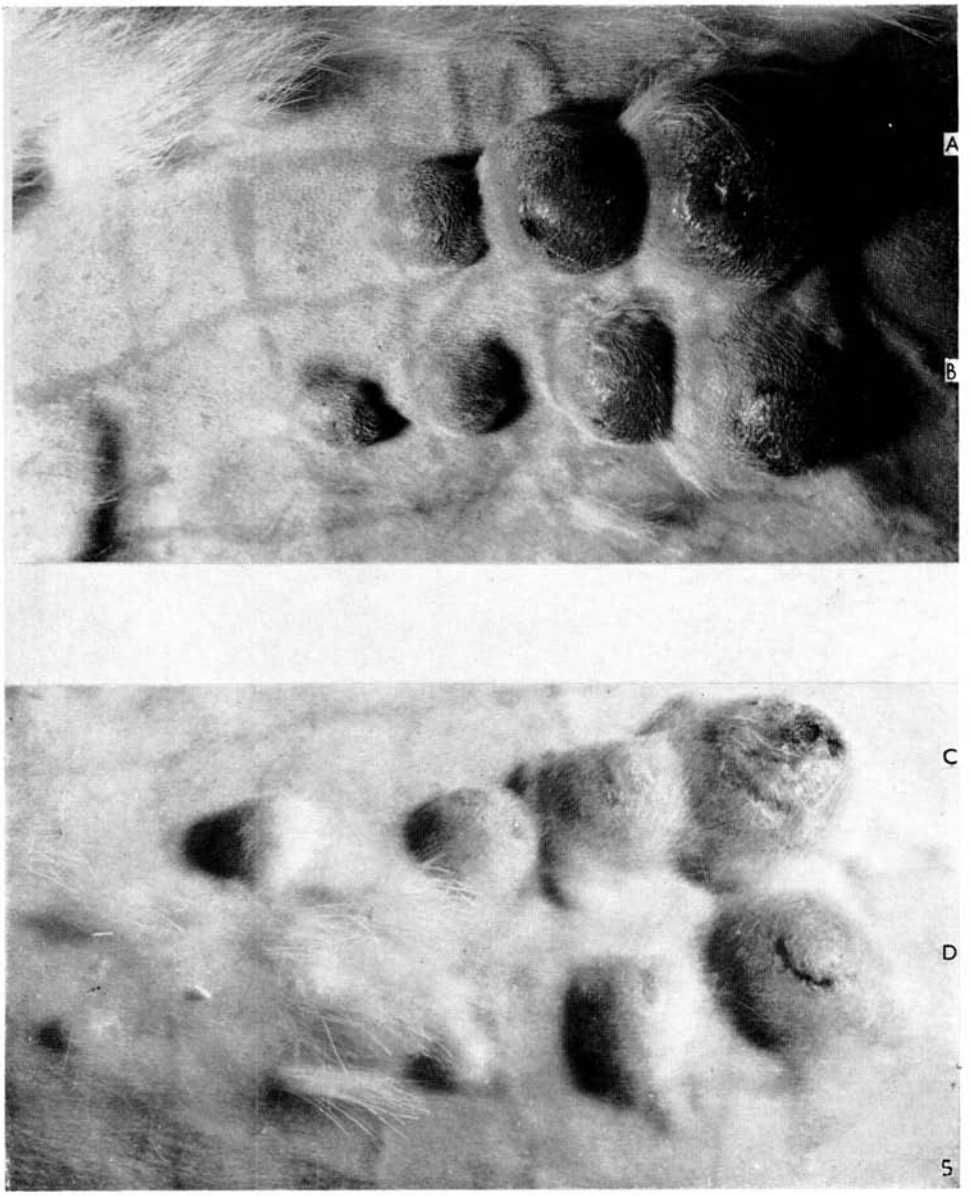

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