FURTHER STUDIES ON THE MECHANISM OF MOSQUITO TRANSMISSION OF MYXOMATOSIS IN THE EUROPEAN RABBIT

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(With Plates 5 and 6, and 2 Figures in the Text)

There is now a considerable body of evidence, experimental and epidemiological, incriminating mosquitoes as important vectors of myxomatosis. In an examination of the transmission of myxomatosis by the mosquito, *Aedes aegypti*, Fenner, Day & Woodroofe (1952) produced unequivocal evidence that the virus could be transferred mechanically by the contaminated mouthparts of mosquitoes which had probed through infected skin lesions. They concluded also that this was the only method of transmission, and that multiplication of the virus in the mosquito *A. aegypti* did not occur.

Subsequently Kilham and co-workers investigated the transmission of the related virus of Shope's fibroma in the cottontail rabbit (Kilham & Woke, 1953; Kilham & Dalmat, 1955). They demonstrated the importance of mechanical transmission of this virus by mosquitoes, but suggested that multiplication of the virus in the mosquito may also occur. They based this suggestion on three lines of evidence:

(*a*) Titrations of the heads of mosquitoes which had fed through an infective fibroma showed a sharp drop in virus content a few days after the acquisition feed, and then a slow rise over several weeks.

(*b*) In one experiment mosquitoes which failed to transmit one or two weeks after the transmission feed did so after about 3 weeks, suggesting a latent period between the acquisition feed and the ability to transmit the virus.

(*c*) In another experiment (Kilham & Woke, 1953) the lesions produced by mosquitoes feeding 1, 4 and 7 days after the acquisition feed differed in character from those produced by immediate interrupted feeding or by refeeding after 4 weeks.

Kilham & Dalmat suggested that, in addition to simple mechanical transfer, fibroma virus may multiply inside the head of the mosquito, being liberated therefrom during later blood meals.

Some experiments have also been reported on the transmission of European myxomatosis by mosquitoes, and the ability of some mosquitoes to transmit for

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several days after the acquisition feed has led Jacotot, Toumanoff, Vallée & Virat (1954) to postulate multiplication of the virus in the mosquito vector.

Mosquito transmission is of major importance in the epidemiology of myxomatosis in Australia. The results reported by Kilham & Dalmat with fibroma virus made it desirable to re-investigate the transmission of myxomatosis by mosquitoes, paying particular attention to the possibility that the virus might multiply in the mosquito. Studies on *Aedes aegypti* were extended to include an important field vector of myxomatosis in Australia—*Anopheles annulipes* Walk. In addition, quantitative methods were devised to facilitate the investigation of the relative transmissibility of different strains of myxoma virus by mosquitoes (Fenner *et al.* 1956).

MATERIALS AND METHODS

Materials

Virus. The standard laboratory strain of myxoma virus, derived from that of Moses (1911), was used in all except two experiments, in which use was made of the attenuated Uriarra strain of Mykytowycz (1953) and the Lausanne strain of Bouvier. The OA and Boerlage strains of fibroma virus were kindly supplied by Dr R. E. Shope.

Rabbits. The rabbits were bred at the Australian National University Animal Breeding Establishment and were used at the age of 4 months. Albino rabbits only were used for experiments involving probing by mosquitoes to facilitate accurate observation of the behaviour of the insects.

Eggs. 11–12-day-old embryonated hen's eggs of White Leghorn stock were used.

Mosquitoes. The majority of experiments were conducted with *Aedes aegypti* (L.), derived originally from a strain from Rockhampton, Queensland, supplied by Mr P. R. Wilkinson, Commonwealth Scientific and Industrial Research Organization. They were bred and maintained at the Division of Entomology, C.S.I.R.O., Canberra, in an insectary kept at about 27° C. and 60–80 % relative humidity. In a few experiments *Anopheles annulipes* and *Aedes alboannulatus* Macq. were used. They were collected as larvae in the country around Canberra and raised in the laboratory at room temperature (19–26° C.).

Diluents. Buffered isotonic saline containing 0·5 % gelatin was used for diluting out virus suspensions. When potentially contaminated material was used—e.g. mosquitoes, skin—penicillin and streptomycin were added to final concentrations of 500 units per ml. and 4 mg. per ml. respectively.

Methods

Virus titrations. Preparations were usually assayed for their virus content by the chorioallantoic inoculation of 11- or 12-day-old developing chick embryos. Sometimes titrations were carried out by the intradermal inoculation of rabbits. The accuracy of these counting methods, and the relative susceptibility of eggs and rabbits, has been discussed elsewhere (Fenner & McIntyre, 1956). The mean number of infective virus units in a given amount of inoculum calculated from the intradermal inoculation of rabbits was 2·5 times greater than the mean pock count

for the same amount of inoculum on the chorioallantoic membrane. Throughout this and the succeeding paper the titres of myxoma virus have been expressed as the number of rabbit-infectious doses, using 2·5 as the conversion factor where necessary.

Mosquito feeding. Several methods were used to infect mosquitoes. In some experiments rabbits were anaesthetized by the intravenous inoculation of 'Veterinary Nembutal' (Abbott) and placed with their scarified backs in contact with the top of a gauze cage containing the mosquitoes, in such a way that the area of back in contact with the cage consisted of myxomatous lesions. Blood-fed mosquitoes were removed at the completion of the feeding period. In other experiments mosquitoes were tubed individually in glass vials 2 × 5 cm., with gauze on one end, and were induced to probe or take a blood feed, whichever might be required in any particular experiment. For the experiments involving repeated probings by individual potentially infected mosquitoes, the shaved backs of white rabbits were marked with a checkerboard pattern, usually eight rows across and twelve to fifteen along the back of a rabbit. The tubed mosquito could then be moved from one square to the next as soon as it had probed satisfactorily. Between 96 and 120 individual probings could thus be made on the back of a single rabbit. Anaesthetization of the rabbit with nembutal greatly simplified this procedure. The type of result obtained is shown in Pl. 5.

Dissection of mosquitoes. Mosquitoes were dissected into head, thorax and abdomen by cutting through the regions of maximum constriction with a triangular cutting needle or a fine scalpel. The thorax therefore contained the salivary glands and portions of the midgut and the diverticula.

Grinding methods

Grinding mosquitoes for virus recovery. Comparisons were made of the efficiency of recovery of virus from preparations containing the heads and mouthparts of twenty mosquitoes ground by four different methods. The grinding vessels were either small porcelain mortars and pestles (6 cm. diameter) or round-bottomed glass tubes 2·5 cm. long and 1 cm. in diameter, with glass pestles which had been ground to fit. Tests were made with or without the addition of a small amount of sterile alundum. In the first experiment 0·05 ml. of a preparation of virus-containing fluid was added to each preparation of twenty normal heads of *Aedes aegypti* before grinding began; in the second experiment groups of twenty mosquitoes were induced to probe through the 7-day-old skin lesions of a rabbit infected with myxoma virus before the head was removed by dissection. In the second group 0·05 ml. of gelatin saline was added to each container. All grinding was in a chilled mortar, which was placed, when grinding was complete, in a deep-freeze chamber until the liquid froze. The thawing deposit was then re-ground, suspended in 1·0 ml. of diluent, and titrated in eggs (Expt. I) or in rabbits (Expt. II). The results are summarized in Table 1. Both experiments indicated the superiority of the small glass grinders, used preferably without added alundum, and this method was adopted as the standard technique in all subsequent experiments.

Grinding skin for virus recovery. Rabbit skin is tough and difficult to grind, and good virus recovery from infected tissue can be expected only if all virus-containing cells are disrupted. The method adopted in obtaining the virus titre of infected skin was governed by these considerations, and by the belief that the part of the skin which chiefly contaminates the mosquito's proboscis is the 1–2 mm. of thickened epidermis over the skin lesions. With a new scalpel blade a skin slice was obtained which comprised only epidermal cells. This was weighed on a torsion balance and then ground with a very small amount of sterile alundum in a small (6 cm. diameter) chilled porcelain mortar and pestle. After preliminary grinding, unhampered by the presence of dermal collagen, the mortar was replaced in the deep-freeze chamber until the paste froze. The paste was then ground again and suspended in an appropriate volume of diluent, the ice crystals aiding the grinding. It was difficult to devise an experiment to test the loss of virus on grinding, but an attempt was made to ascertain the reproducibility of the procedure. Approximately equal-sized skin slices were taken from the summits of eight local lesions

Table 1. *Comparison of the efficiency of different methods of grinding the virus-contaminated heads and mouthparts of mosquitoes*

Method of grinding 20 heads of *Aedes aegypti*	Expt. I, 0·05 ml. virus suspension added to each preparation	Expt. II, mosquitoes probed through myxoma lesions
Small mortar and pestle: with alundum	70*	1
Small mortar and pestle: without alundum	1150	10
Small glass grinders: with alundum	2100	50
Small glass grinders: without alundum	2300	125

* Expressed as rabbit-infectious doses per ml. of suspension.

produced by the inoculation of aliquots of a high concentration of virus in the skin of a rabbit. Different slices weighed between 40 and 71 mg. (mean 53 mg.). They were ground as described above, taken up as 10 % (w/v) suspensions and inoculated on eggs at a dilution of 10^{-3}. The results are recorded in Table 2.

There was considerable variation in the titres obtained under standardized conditions. The differences observed might reflect differences in the actual virus content of the tissues, the efficiency of extraction of virus, or the variable loss of virus due to absorption or inactivation during the grinding process. The major variation between samples arose from the high titres obtained with samples III and VIII, and the low titre in sample IV. The titres of the other five samples were reasonably homogeneous, and if we consider that the mean titre of these five (9·1) was the best approximation to the mean titre of skin slices for such lesions, five of the eight samples approached that figure closely, one was only one-sixth of it, one was four times and one eight times larger.

The reproducibility of results obtained by grinding the heads and mouthparts of infection mosquitoes. It was shown earlier that the highest virus recovery was obtained after grinding contaminated mouthparts in a small glass grinder without added abrasive. When comparisons had to be made between the virus content of

these organs from mosquitoes which had fed through the skin lesions of rabbits at various intervals after infection, additional problems were encountered. Experiments to be described later have shown that there is considerable variation in the virus load on the mouthparts of individual mosquitoes, and that some can fail to take up any virus. There are also variations in the virus content of the skin of rabbits infected by scarification of the back, or by intradermal inoculation, and there are potentially variations in the reproducibility of the grinding operation, as well as in the actual titration procedures. Two experiments were conducted to assess the total variability of mosquito pools—one using the heads and mouthparts of sixty mosquitoes in each batch and one using those of fifteen mosquitoes per

Table 2. *Results of the titration on eggs of eight tissue slices obtained from eight lesions produced on the back of a rabbit by the inoculation of* 0·1 *ml. volumes of* 5×10^4 *rabbit-infectious doses of myxoma virus* 6 *days earlier*

	Sample no.							
	I	II	III	IV	V	VI	VII	VIII
Weight (mg.)	58	43	71	45	61	59	40	51
Volume of diluent (ml.) ...	0·6	0·4	0·7	0·5	0·6	0·6	0·4	0·5
Pock counts (individual eggs)	11	7	14	2	11	7	9	78
	16	40	42	1	11	7	5	60
	8	7	33	0	17	3	4	131
	14	23	13	4	6	11	13	19
	7	4	85	1	9	3	6	99
	5	14	52	2	7	0	5	89
	4	2	35	0	3	11	5	29
	12	22	13	2	0	—	7	—
Mean	9·6	15	36	1·5	8	6	6·8	72
Mean I–VIII incl.	19·4	—	—	—	—	—	—	—
Mean I, II, V, VI, VII	9·1	—	—	—	—	—	—	—

batch. In the first experiment, the mosquitoes were allowed to probe through the back of a rabbit scarified 5 days earlier; in the second the mosquitoes were fed individually in glass tubes on 7- and 8-day-old lesions produced by intradermal inoculation. Titrations were carried out in eggs and rabbits. Only the results of egg titrations are given in Table 3.

Fewer sources of error enter into the estimation of the virus titre of mosquitoes or parts thereof than of tissue slices. Grinding does not require great force, as is required to break down skin slices, and glass grinders without alundum can be used. There are, of course, variations in the acquisition of virus by mosquitoes. The results show that with both feeding techniques reasonably uniform results were obtained, except for one batch (D of Expt. I) which had only one-quarter of the virus content of the other three batches of that experiment.

EXPERIMENTAL RESULTS

The localization of virus in the mosquito

Previous experiments (Fenner *et al.* 1952) indicated that virus imbibed in blood played no part in the transmission of myxomatosis but was excreted in the faeces of the infected mosquito. In adult domestic rabbits infected with fibroma virus,

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viraemia is transient (Andrewes, 1936; Hurst, 1937). Kilham & Fisher (1954) were unable to demonstrate viraemia in cottontail rabbits infected with fibroma virus, either by subinoculation of blood or by allowing mosquitoes to take a blood meal on the uninvolved skin of infected cottontails. All available evidence with myxoma and fibroma viruses, as with fowlpox, indicates that the infected skin

Table 3. *The reproducibility of estimations of the virus load acquired by mosquitoes, tested by grinding their heads and mouthparts in small glass grinders*

	Expt. I, 60 mosquitoes per group; fed in cage on scarified rabbit					Expt. II, 15 mosquitoes per group; fed individually		
	Pock counts							
Group ...	A	B	C	D		E	F	G
Dilution ...	10^{-1}	10^{-1}	10^{-1}	10^{0}	10^{-1}	10^{0}	10^{0}	10^{0}
	14	11	10	31	7	10	11	7
	14	33	6	54	1	3	4	10
	15	7	23	28	3	2	10	7
	11	—	35	20	—	3	8	3
	13	—	10	46	—	5	7	5
				67	—	11	9	17
				25	—	6	7	3
				14	—	4	4	6
						3	19	8
						0	2	3
Mean	13·4	17·0	16·8	35·5	3·7	4·7	8·1	6·9

Table 4. *Analysis of variance of data given in Table* 3

	Degrees of freedom	Sum of squares	Mean square	*F*	Significance
Expt. I (A, B, C only):					
Treatment	2	37	18·5	< 1	N.S.
Error	10	980	98·0	—	—
Expt. II:					
Treatment	2	59	29·5	1·68	N.S.
Error	27	476	17·6	—	—

Expt. I—comparison of D 10 with A, B, C:

D 10^{0}/10 mean = 3·563. Variance of mean = 0·417.

A, B, C mean = 15·538. Variance of mean = 7·538.

$$\frac{\text{Difference of means}}{\text{S.E. of difference}} = \frac{11{\cdot}965}{2{\cdot}820} = 4{\cdot}24, \quad P < 0{\cdot}001.$$

lesions are the only source of virus capable of rendering mosquitoes infectious. The question arises therefore of the location in the mosquito of the virus acquired by mosquitoes feeding through infected lesions.

Kilham & Dalmat (1955) reported experiments on the presence and persistence of fibroma virus in various tissues of *Aedes aegypti* mosquitoes which had fed on fibromas on cottontail rabbits. Whereas no virus was demonstrable in the thorax and abdomen, virus in or on the head and mouthparts persisted throughout the

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35 days of the experiment. The maximum virus content was recorded 1 day after the infective feed.

A comparable experiment was performed with myxoma virus. About 100 *A. aegypti* were fed through the skin lesions of a rabbit inoculated intradermally. The superficial layers of the skin of these lesions were ground up and titrated, and the virus content was found to be about 10^8 rabbit-infectious doses per g. Heparinized blood contained about $10^{5\cdot5}$ rabbit-infectious doses per ml. Immediately after the infective feed fifteen mosquitoes were killed by being placed in a deep-freeze cabinet, and then dissected into head and mouthparts, thorax and appendages, and abdomen. These portions were pooled, ground in small glass grinders, without abrasive, and taken up in 1 ml. of diluent. Serial dilutions of these suspensions were titrated in eggs and rabbits. The same procedure was repeated 2 days after

Table 5. *The persistence of myxoma virus from various parts of* Aedes aegypti *which had fed through skin lesions containing* 10^8 *rabbit-infectious doses per g., and on blood containing* $10^{5\cdot5}$ *rabbit-infectious doses per ml. Pools of fifteen mosquitoes. Results expressed as numbers of rabbit-infectious doses per mosquito (or part thereof). Data on fibroma taken from Kilham & Dalmat* (1955)

Time between infective feed and titration (days)	Head and mouthparts		Thorax and limbs		Abdomen	
	Fibroma	Myxoma	Fibroma	Myxoma	Fibroma	Myxoma
0	—	130	—	5	—	500
1	1000	—	0	—	0	—
2	—	80	—	6	—	6
4	1	—	0	—	0	—
7	—	16	—	0	—	0·1
9	0·1	—	0	—	0	—
14	1	0·5	0	2	0	0·2
22	—	0·5	—	0	—	0
27	1	—	0	—	0	—
28	1	3	0	0	0	0
35	10	—	0	—	0	—

the infective feed. The remaining mosquitoes were released into a cage kept in an insectary at 27° C. and about 60 % relative humidity, and fed on sugar and water. At weekly intervals groups of fifteen mosquitoes were removed, dissected, ground and titrated. Immediately after the removal of each group the remaining mosquitoes were allowed to feed on a mouse. The results are shown in Table 5.

A considerable amount of virus was taken into the abdomen with the infected blood, but the concentration fell rapidly and negligible amounts were found after the second day. Virus was recovered in very small amounts and irregularly in suspensions of the thorax plus appendages, probably due to the inclusion of portions of the midgut in the thorax. Virus persisted for longer on the head and mouthparts, positive results being obtained throughout the experiment, although concentrations were very low after the end of the first week. The results reported by Kilham & Dalmat (1955) are not as different as appears from a cursory examination of their Table 1, and we have inserted calculations of the numbers of infectious

particles made on the same basis as our own into our Table 5. When the inaccuracies of calculating an end-point by the presence or absence of a lesion in the single inoculation made with tenfold dilutions of a suspension of virus, together with possible variations due to grinding, dilution and sampling errors, are taken into account, it is apparent that with fibroma virus there is initially a relatively high concentration on the head and mouthparts; and that this falls off rapidly and then remains approximately constant for 5 weeks. The early presence of virus in the abdomen and thorax with myxoma virus and its absence with fibroma reflect the high viraemia associated with myxomatosis and the absence of demonstrable viraemia in fibroma infections.

An attempt was made to determine whether virus associated with the pooled heads and probosces in the previous experiment was located on the proboscis or the head, or in both. Groups of about 100 *A. aegypti* were allowed to probe several times and then take a blood meal, through the skin lesions of infected rabbits. The heads were removed from the bodies of twenty-five of them, and the

Table 6. *The distribution of myxoma virus on the head and mouthparts of* Aedes aegypti *mosquitoes infected by probing and feeding through the skin lesions of rabbits inoculated intradermally 7 days earlier*

Expt. nos.	Time between infective feed and titrations (days)	Titre of virus (rabbit-infectious doses per mosquito part)	
		Head	Proboscis
I	0	1·2	0·2
II	0	15·8	2·2
	3	0	0·2
III	0	2·5	0·3
	3	0·2	4·6

proboscis severed as near to the head as possible. Precautions were taken to avoid contamination of either part with virus from another part of the mosquito. The pooled heads and pooled probosces were ground without abrasive in small glass grinders, suspended in 1·5 ml. of diluent and inoculated into rabbits and eggs. The titrations were repeated 3 days later. The results of three such experiments are shown in Table 6.

Although immediately after the acquisition probe virus was recovered from both the head and mouthparts, and in higher concentration from the head, 3 days later the concentration of virus was greater in the proboscis.

It was thought that the virus recovered from the head of the mosquitoes might derive from virus-containing fluid imbibed by the mosquito, and be of no significance in transmission. A possible test of this would be to demonstrate virus in the head (but not on the proboscis) of mosquitoes which had taken a blood feed on a viraemic rabbit from a site and at a time where there was no virus localized in the skin. Sixty *A. aegypti* mosquitoes were induced to feed on the ears of a rabbit which had been inoculated intradermally 5 days earlier. The heparinized blood of this rabbit contained $10^{5·3}$ rabbit-infectious particles per ml. of virus. Immediately

after the blood feed and again 3 days later, the head and proboscis of twenty-five mosquitoes were dissected from each other and from the rest of the body and titrated. No virus could be recovered from the head or mouthparts of any of the mosquitoes, although the guts contained appreciable amounts of virus imbibed with the virus-containing blood. The results confirm previous experiments (Fenner *et al.* 1952), and indicate that virus in the blood does not contaminate the head and mouthparts, but passes immediately to the midgut.

Injection of virus into the haemocoele

All the foregoing experiments are in agreement with the view that the significant virus, from the point of view of transmission, was located in or on the head and mouthparts. An experiment was made to determine whether virus introduced directly in the haemocoele of *A. aegypti* might multiply there.

About 100 mosquitoes were inoculated into the haemocoele with small volumes (approximately 0·001 ml.) of a suspension of myxoma virus which contained approximately 10^8 rabbit-infectious doses per ml. The mosquitoes were maintained on sugar and water at 27° C. and 60 % relative humidity. At intervals, groups of

Table 7. *Persistence of myxoma virus after inoculation into body cavity of* Aedes aegypti

Time after inoculation (days)	No. of mosquitoes tested	Rabbit-infectious doses per mosquito
1	5	420
3	10	1·6
5	10	1·6
7	10	0
11	10	0
17	38	0

mosquitoes were ground without abrasive in small glass grinders, taken up in 1 ml. of diluent and inoculated into rabbits and eggs. The results, shown in Table 7, demonstrate the rapid disappearance of virus from the mosquito, and the absence of multiplication of the inoculated virus.

Kilham & Dalmat (1955) repeatedly failed to obtain transmission of fibroma by the inoculation of virus suspensions into the haemocoele of *A. aegypti*. McLean (1955) demonstrated the multiplication of encephalitis (MVE) virus in the haemocoele after injection, but the failure of vaccinia, herpes simplex and influenza viruses to multiply after injection into the haemocoele of *A. queenslandis* and *Culex annulirostris*. No experimental evidence is available therefore, to suggest that any of the three pox viruses examined multiplies after inoculation into the haemocoele of mosquitoes.

The ability of mosquitoes to transmit myxomatosis at intervals after repeated probes

In order to determine whether virus located elsewhere than on the contaminated mouthparts could cause infection of a rabbit, an experiment was conducted in which mosquitoes which had cleansed the proboscis of infectious virus by repeated

probing of a normal rabbit were tested for their ability to transmit myxomatosis subsequently.

Fifty mosquitoes were allowed to probe through the skin lesions of a rabbit inoculated 7 days earlier with myxoma virus (skin titre about 10^7 rabbit-infectious doses per g.). Two days later about half of them were induced to probe repeatedly on the back of a normal rabbit, with the intention of 'wiping off' virus which adhered to the mouthparts. At various intervals thereafter groups of these mosquitoes, and of the other mosquitoes which had not been subjected to the cleansing procedure, were allowed to probe several times on marked skin sites on normal rabbits. Four days after the initial infective probing the mosquitoes were released into a cage, and given daily opportunities to obtain a blood meal from a mouse. The mosquitoes were kept in the insectary, at 27° C. and 60 % relative humidity, throughout. The results are shown in Table 8.

Table 8. *The persistence of virus in mosquitoes which had probed through the skin lesions of a myxomatous rabbit and then been allowed to probe many times through the skin of normal rabbits*

Time between infective feed and probing experiment (days)	Mosquitoes subjected to cleaning of proboscis		Control mosquitoes
2	After first probe	19/24*	—
	After 2nd–19th probe	15/24	—
	After 20th probe (blood meal)	10/24	—
4	After single probe per site	6/15	—
11	After 5 probes per site	1/12	—
18	After blood meal	0/14	5/30
26	After blood meal	0/11	0/20

* Numerator = number of mosquitoes producing infection on probing (feeding); denominator = total number of mosquitoes probing (feeding).

The numbers of mosquitoes available for test from the eighteenth day onwards were too small to detect statistically significant differences between the mosquitoes which had cleansed the proboscis and control insects. However, no positive results were obtained with the mosquitoes which had 'wiped off' the virus which contaminated their mouthparts, and the overall trend of infections with time showed a steady decline—19/24 on day 2, falling steadily to 5/44 at day 18 and 0/31 on day 26. These results are consistent with mechanical transmission by contaminated mouthparts and give no indication of multiplication of the virus in the mosquitoes up to 26 days after the infective feed.

Failure to obtain evidence of multiplication of myxoma virus in Aedes aegypti *and* Anopheles annulipes

The previous experiments failed to demonstrate multiplication of myxoma virus in *Aedes aegypti* in the first 26 days after the acquisition feed. It might be argued, however, that *A. aegypti*, although such a convenient laboratory insect, is not a natural vector, and that multiplication could occur in some other species of

mosquitoes which can function as natural vectors. Further experiments designed to demonstrate the existence of multiplication of the virus in the mosquito, if it did indeed occur, were carried out with *A. aegypti* and also with *Anopheles annulipes*, one of the important natural vectors in Australia.

Total numbers of 180 *Aedes aegypti* and 160 *Anopheles annulipes* were induced to take blood meals through the backs of three rabbits which had been scarified and infected with myxoma virus six days previously. The blood titres of these rabbits were $10^{5\cdot3}$, $10^{5\cdot8}$ and $10^{6\cdot1}$ rabbit-infectious doses per ml., and the concentrations of virus in the epidermal layers of the skin of the backs $10^{7\cdot1}$, $10^{7\cdot5}$ and $10^{8\cdot4}$ rabbit-infectious doses per g. The mosquitoes were maintained on sugar and

Table 9. *The persistence of myxoma virus in* Aedes aegypti *and* Anopheles annulipes *after feeding through the skin lesions of rabbits which were circulating high titre virus. Mosquitoes maintained on sugar and water at* 18–22° C.

Species of mosquito	Time between infective and subsequent feeds (days)	Nos. of mosquitoes	Inoculation in rabbits 10^{0}	10^{-1}	10^{-2}*
Aedes aegypti	3	22	9/22†	2/22	0
	8	10	9/10	1/10	0
	15	10	1/10	0/10	0
	22	10	1/10	0/10	0
	29	26 (tested in pairs)	2/13	0/13	0
Anopheles annulipes	3	8	4/8	2/8	0
	8	16 (tested in pairs)	6/8	4/8	1/8
	15	10	3/10	1/10	0
	22	10	0/10	0/10	0
	29	12	2/12	0/12	0

* Tested also at 10^{-3} dilutions—all such tests were negative.

† Numerator = number of infective mosquitoes (or pairs); denominator = total number of mosquitoes (or pairs).

water at room temperature (18–22° C.) throughout the experiment. At various intervals after feeding on the infected rabbits, a number of mosquitoes of each species were individually ground in small glass grinders without alundum, and suspended in 0·5 ml. of diluent. The bulk of this volume, and three serial tenfold dilutions of it in volumes of 0·5 ml., were inoculated intradermally in rabbits, two skin sites being used for each dilution. The results are presented in Table 9, which shows the proportion of individual mosquitoes containing virus at various intervals after the acquisition feed. Virus persisted for as long as 29 days in an occasional mosquito, but there was a steady decline in the proportion of infected mosquitoes, and in the titre of virus in those which were infected. In the group of sixteen *Anopheles annulipes* tested 8 days after the infectious feeding one pool of two insects caused infection at a dilution of 1/100, although none of eight mosquitoes tested individually on the third day caused infection at this dilution. We ascribe this result to the chance occurrence in this group of one insect with a high virus

load. There was no evidence of an increase in the amount of virus, or of an increase in the proportion of infected mosquitoes with the passage of time.

High mortality in the cages of *A. annulipes* interfered considerably with this experiment, and another experiment was therefore carried out with this species. Mosquitoes were induced to feed on the scarified back of an infected rabbit (blood titre $10^{4\cdot7}$ rabbit-infectious doses per ml., skin titre $10^{7\cdot7}$ rabbit-infectious doses per g.). Groups of twenty mosquitoes were ground and tested for the presence of virus immediately after the infective feed and then at weekly intervals. The mosquitoes were maintained on sugar and water at room temperature (18–22° C.) throughout the experiment. Grinding was carried out in the usual way, in small glass grinders without abrasive, and the ground material was suspended in 2·5 ml. of diluent. All titrations were made using serial twofold dilutions of virus with 1·0 ml. volumes, and each dilution of each series was inoculated in 0·1 ml. doses into eight sites on the back of a rabbit. In each series the same rabbit was used for dilutions 1/1 to 1/8, and another for dilutions 1/16 to 1/4096, in order to avoid the early secondary lesions which sometimes complicated readings at the higher dilutions, and to avoid the possibility of suppressing lesions due to the very dilute inocula. The results are recorded in Table 10. They confirm the previous experiment. There was a progressive diminution in the virus titre of the mosquito suspensions, and no evidence whatever of a rise in titre after a latent interval. The solitary positive result on the forty-third day indicates only that at least one virus particle, and probably not many more, had survived in one group of twenty mosquitoes for as long as 43 days at room temperature.

Quantitative aspects of the mechanical transmission of myxoma virus by mosquitoes

It is impossible to prove that myxoma virus does not multiply in mosquitoes, but no evidence of such multiplication was obtained in the series of experiments just described. The remainder of the paper is devoted to the study of quantitative aspects of mosquito transmission of myxoma virus. All the experiments to be described were based on the hypothesis that transmission was solely mechanical in nature, virus being acquired when mosquitoes probed through infected epidermal cells, and susceptible rabbits being infected with virus dislodged from the contaminated mouthparts of an infected mosquito.

Mosquitoes which had probed or fed through the skin lesions of an infected rabbit were induced to probe in succession on a series of marked skin sites on the back of a susceptible rabbit. Anaesthetized albino rabbits were used, the presence or absence of lesions at the site of probing being determined by examination of the rabbits on the third, fourth and fifth days after exposure. A typical result is shown in Pl. 5. Variations of this experimental technique were used to compare the relative transmissibility of different strains of myxoma virus, to determine the rate of loss of infectiousness of mosquitoes due to the passage of time and repeated probings, and to determine the relative efficiency as mechanical vectors of different species of mosquitoes. The comparative results are reported in the succeeding paper.

Table 10. *Titrations of groups of twenty* Anopheles annulipes, *maintained on sugar and water at* 18–22° C., *at intervals after they had fed through the skin lesions of an infected rabbit*

Interval between infective feed and test (days)	Dilution of mosquito suspension												
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
0	8*	8	8	8	8	8	8	8	8	5	2	1	0
7	7	3	3	3	1	0	0	0	0	0	0	0	0
14	3	1	2	0	0	0	0	0	0	0	0	0	0
22	1	1	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0	0	0	0
43	1	0	0	0	0	0	0	0	0	0	0	0	0

* 8, 7, 0, etc. = 8, 7, 0, etc., lesions produced out of eight inoculations of each dilution.

Table 11. *The results of successive probes on the backs of susceptible rabbits by* Aedes aegypti *which had previously probed through skin lesions produced by the intradermal inoculation of a rabbit with* 5×10^4 *rabbit-infectious doses of the Lausanne strain of myxoma virus* 7 *days earlier. The virus titre of the skin lesions was* $10^{7 \cdot 8}$ *rabbit-infectious doses per g. Separate groups of mosquitoes used at* 0, 2, 3 *and* 4 *days. Experiment conducted at* 27° C. *and* 60–80% *relative humidity. Mosquitoes used* 3 *and* 4 *days after infectious probing released into cage and given sugar and water on the second day*

Days after infective probe	Probing number																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Totals
0	20/24*	15/24	13/23	6/23	9/23	10/23	10/22	6/22	8/21	7/21	6/20	4/20	6/20	3/20	2/19	4/19	2/19	4/19	7/18	142/400
2	16/23	13/23	10/23	12/23	9/22	7/22	9/22	8/21	8/21	4/20	0/20	4/19	1/19	2/19	1/19	2/18	5/18	2/15	2/15	115/382
3	14/26	10/24	9/23	7/23	4/22	4/22	9/21	5/20	6/20	5/20	3/19	5/19	0/19	3/18	2/18	3/18	1/18	1/17	0/16	91/383
4	10/22	12/22	10/22	7/22	7/22	6/20	5/20	3/19	1/19	3/19	2/19	2/19	2/18	2/17	4/17	3/16	2/15	1/14	1/13	83/355

* Numerator = number of probes resulting in production of a local lesion; denominator = total number of probes.

Here we will describe the results obtained with *Aedes aegypti* and the Lausanne strain of virus, as this experiment provided a more satisfactory basis for analysis than did experiments performed with the standard laboratory strain.

A donor rabbit was prepared by the intradermal inoculation in four sites of 5×10^4 rabbit-infectious doses of the Lausanne strain of myxoma virus. Seven days later the mean titre of virus in the skin lesions was $10^{7 \cdot 8}$ rabbit-infectious doses per g., and about 150 *A. aegypti* were allowed to probe several times through the lesions. Some were allowed to engorge and these were not used for at least 48 hr. All mosquitoes were kept in individual tubes for 48 hr., and if not used by that time were released into cages and given sugar and water, and were re-tubed before use at 72 and 96 hr. The experiment was conducted in a warm room maintained at 27° C. and 60–80 % relative humidity. Immediately after the infective probing, and 48, 72 and 96 hr. later, about twenty-five mosquitoes were induced to probe up to twenty times on marked skin sites on the backs of susceptible rabbits. Each insect was discarded after it had probed the susceptible rabbit nineteen times. Readings of the rabbits were made 4 and 5 days after the probings. The results are summarized in Table 11. They have been used as a basis of the analysis set out in the appendix. The validity of this analysis depends upon the assumption that

Table 12. *Characteristics of the transmission of the Lausanne strain of myxoma virus by* Aedes aegypti

Median minimal initial virus load (rabbit-infectious doses)	Amount of virus lost on successive probes (percentage of load per probe)	Amount of virus lost per day (percentage of load)
7·8	12	20

there is a constant probability that an infectious unit dislodged from the proboscis of a mosquito will cause infection recognizable by the development of a skin lesion, and that a constant proportion of the material present on the proboscis is dislodged on each probe. The results of the analysis presented in the appendix indicate that these assumptions are approximately if not exactly true. The salient characteristics of the mosquito transmission of the Lausanne strain of myxoma virus, derived from this analysis, are presented in Table 12.

Location of the virus particles in infected skin

A feature of the type of experiment just described, noticeable particularly in experiments made with strains other than Lausanne, was the large number of mosquitoes which failed to become infective. Efforts to reduce the proportion by permitting mosquitoes to probe three or four times through different parts of the tumours used for the acquisition 'feed' were only partially successful.

An attempt was made to observe the distribution of elementary bodies of the virus in sections stained with fluorescent antibody, using the techniques described by Coons & Kaplan (1950). High-titre serum was obtained from rabbits which had recovered from myxomatosis. The globulin fraction obtained by ammonium sulphate precipitation was coupled with fluorescein isocyanate, and the coupled

antibody extracted with dried mouse liver. Fragments of myxoma lesions were fixed overnight in neutral formalin, dehydrated in alcohol, cleared in xylol, and embedded in paraffin. Sections 10μ thick were treated with the fluorescent-coupled antibody. Freeze-dried tissues were also sectioned but proved less satisfactory than formalin-fixed material.

Autofluorescence of the hair was easily distinguished from localized fairly intense staining of certain groups of cells in the dermal tissues. The latter but not the former staining was reduced by pretreatment of the sections with high titre non-coupled antibody. These groups of cells were distributed very irregularly and their exact relationship to other tissue elements could not be determined. Nuclei of unstained cells surrounding the stained cells occasionally exhibited some fluorescence.

Epstein, Reissig & De Robertis (1952) have demonstrated many elementary bodies of myxoma virus in the cytoplasm of cells by electron-microscopy of thin sections of tumours, but their descriptions refer to cells deep in the tissue, and therefore not accessible to mosquitoes.

Further studies using both fluorescent antibody and electron microscopy are required before exact information is available on the distribution of virus in the cells which constitute the skin lesions, but the information available suggests that the irregular distribution of virus-containing cells would account for the irregularities observed in the acquisition of virus by mosquitoes.

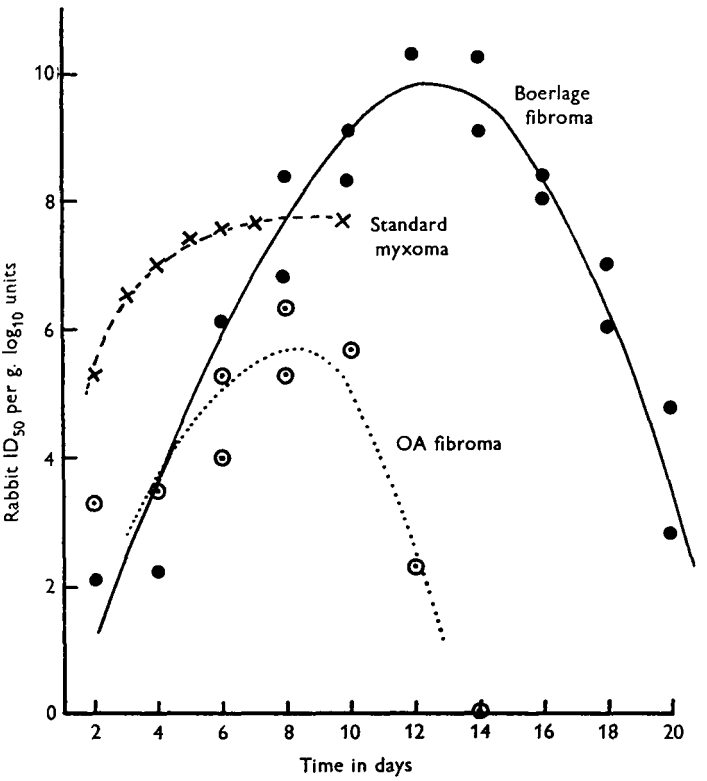
The availability of virus in the skin cells of infected rabbits

Experiments with fibroma virus illustrate well the importance of the availability of virus in determining the efficiency of mechanical transmission by mosquitoes. Kilham & Dalmat (1955) were unable to transmit fibroma virus from one domestic rabbit to another by *Aedes aegypti* fed on the tumours on the twelfth, fourteenth and twentieth days. Our own very extensive trials were scarcely more successful. We obtained only five tumours in domestic rabbits out of 1627 bites by 619 mosquitoes, which had probed through tumours produced by the Boerlage strain of fibroma virus. The successful transmissions occurred on the twelfth, thirteenth and fourteenth day after inoculation of the rabbit.

This result is all the more surprising when the high virus titre of the epidermis over the fibromas is considered. Titration of the skin lesions induced by the injection of doses of 100 rabbit-infectious doses of the OA and Boerlage strains of fibroma virus gave the results shown in Text-fig. 1. The curve obtained with the standard laboratory strain of myxoma virus is included for comparison. In spite of the very high concentration of virus in the superficial layers of the skin in infections with Boerlage fibroma virus, infection of an *A. aegypti* mosquito appears to have been a very rare event, even when allowance is made for the fact that fibroma virus infection by mosquitoes sometimes occurs without the development of a detectable tumour (unpublished observations). This contrasts sharply with the frequent transmission of myxomatosis, the virus titres of which were considerably lower.

In contrast, Kilham & Dalmat (1955) found that tumours on adult cottontail

rabbits, with apparently much lower concentrations of virus than those described above, were rich sources of virus for the *A. aegypti* mosquito, if the tumour was at least 35 days old. These authors ascribe this difference between domestic and cottontail rabbits, and the failure of adult representatives of the latter species to be infective for the first 4 weeks after infection, to the necessity for 'maturation' of the virus in the skin. This appears to be a reasonable explanation, if by maturation is understood a localization of the virus which makes it accessible to the probing mouthparts of a mosquito. Studies of the changes in intracellular localization of the fibroma virus in cottontail tumours, perhaps with fluorescent antibodies or by



Text-fig. 1. The titre of virus ($\log_{10} ID_{50}$) in slices of skin taken from the surface of lesions produced by the intradermal inoculation of rabbits with the OA and Boerlage strains of fibroma virus, and the standard laboratory strain of myxoma virus.

electron microscopy of the epidermal cells, might provide conclusive evidence of the nature of maturation. Electron microscopy of Shope fibromas in both domestic (Bernhard, Bauer, Harel & Oberling, 1955) and cottontail rabbits (Lloyd & Kahler, 1955) showed numerous elementary bodies in infected cells, but the relation of these cells to the skin surface, and thus their availability to the mosquito, could not be determined from the published photographs.

In myxomatosis the regression of the lesion in infections with neuromyxoma, the only strain with no appreciable mortality after infections with small doses of virus, is too rapid to allow maturation to be a factor of any importance. In

infections with highly virulent strains the progress of the disease is too rapid, and the interval between appearance of the lesion and death of the rabbit too short for maturation to occur.

An experiment was therefore performed with the most attenuated strain yet recovered from the field in Australia, the Uriarra strain of Mykytowycz (1953). Four rabbits were infected over the back by scarification with strain Uriarra III. On the fifth, seventh, fourteenth and twenty-first days the concentration of virus in skin slices was determined, and groups of 40–100 *A. aegypti* were allowed to take blood feeds through the skin of the anaesthetized rabbits. The head and proboscis were cut from the body of each engorged mosquito, and two or three pools were made, each containing between twelve and fifty mosquitoes, according

Table 13. *The titre of virus in the skin, and the average virus load on the mouthparts of mosquitoes fed on the scarified backs of rabbits infected with the Uriarra III strain of myxoma virus, at various intervals after infection*

Days after infection of rabbits	Material	Rabbit no.* 576	577	578
5	Skin slice	$10^{6\cdot5}$†	$10^{7\cdot6}$	—
	Mosquitoes: A	13·5‡	6·6	—
	B	11·4	7·7	—
	C	7·9	19·0	—
7	Skin slice	$10^{7\cdot0}$	$10^{8\cdot0}$	—
	Mosquitoes: A	6·6	5·8	—
	B	1·6	4·7	—
14	Skin slice	—	$10^{8\cdot6}$	$10^{8\cdot6}$
	Mosquitoes: A	—	7·5	18·1
	B	—	9·5	13·5
21	Skin slice	—	—	$10^{7\cdot8}$
	Mosquitoes: A	—	—	4·3
	B	—	—	1·1
	C	—	—	0·7

* Rabbit 576 died on the thirteenth day, rabbit 577 on the eighteenth day and rabbit 578 on the twenty-seventh day after scarification.

† Rabbit-infectious doses per g. tissue.

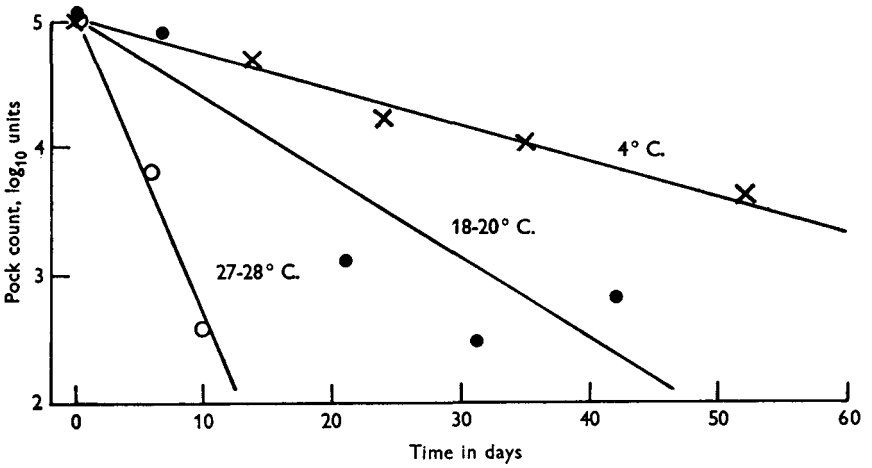
‡ Rabbit-infectious doses per mosquito.

to the numbers available. These were ground in small glass grinders without abrasive, taken up in 0·5 ml. of diluent, and titrated on the chorioallantois and intradermally in rabbits. The results are recorded in Table 13. The average virus load per mosquito did not vary greatly throughout the experiment. With this strain of myxoma virus, chosen as the one in which an effect of maturation of the lesions on infectiousness for the mosquitoes might be most readily demonstrable, there was a possible maximum infectiousness on the fourteenth day, which may be correlated with the fact that the maximum skin concentration of virus was not attained until then. On the twenty-first day both the skin titre and the average virus load per mosquito had fallen appreciably. There appears to be no evidence, in myxomatosis of the European rabbit, of the type of maturation of the lesion which apparently occurs in fibroma of cottontail rabbits, that is, some change in

the distribution or perhaps the nature of the virus particles rather than in their concentration.

The survival times of myxoma virus at different temperatures

The survival time of myxoma virus prepared from ground rabbit-lesion material, and suspended in normal rabbit serum, was determined by periodic titrations on the chorioallantois of the contents of tightly stoppered bottles stored at 4° C., at room temperature (18–20° C.) and at the temperature of the insectary (27–28° C.). The results are shown in Text-fig. 2. At 4° C. the half-life of the virus was about 11 days, at room temperature about 5 days, and at the temperature of the insectary about 31 hr.



Text-fig. 2. The decline in viability of myxoma virus suspended in normal rabbit serum and kept at refrigerator temperature (4° C.), room temperature (18–20° C.), and insectary temperature (27–28° C.). Virus concentration expressed as pock count on the chorioallantois, in $\log_{10}$ units.

DISCUSSION

The work reported in this paper had two main objectives; to elucidate the mechanism of transmission of the myxoma virus by mosquitoes, and to establish a quantitative measure of mosquito transmission, in order to permit comparison between different strains of myxoma virus, and different species of mosquito.

The following lines of evidence bear on the problem of the transmission mechanism:

(1) Virus is acquired from skin lesions and not from blood. Virus in the mosquito midgut does not induce infection (Fenner *et al.* 1952).

(2) Virus injected into the haemocoele does not multiply.

(3) There is no latent period between acquisition of virus and the ability to transmit, and interrupted feeding usually causes infection.

(4) Virus concentration in the mosquito decreases with time.

(5) The probability that a mosquito will cause infection decreases with each probe.

(6) There is no vector specificity.

Each of these points contrasts with similar data obtained in studies with the viral encephalitides. In these infections other workers (for references see Day, 1955) have established the following facts:

(1) Infection of the vector is established by the ingestion of viraemic blood.

(2) Virus inoculated into the haemocoele multiplies in the vector.

(3) After the acquisition feed there is a latent period, during which bites by the mosquito do not result in infection, and interrupted feeding very rarely causes infection.

(4) After the latent period the concentration of virus in the mosquito rapidly attains its peak level, and thereafter does not greatly decrease with time.

(5) The probability of infection does not decrease with succeeding probes.

(6) Vector specificity is marked.

Thus the transmission of myxoma virus differs in every particular from the transmission of the viral encephalitides, and each point of difference indicates that the virus involved in the transmission of myxomatosis has not undergone multiplication in the vector.

Several of the experiments reported were designed to demonstrate directly multiplication of myxoma virus in the mosquito vector, should this occur. The results of all of them were negative, and all our data are compatible with the hypothesis that transmission is purely mechanical. There can be little doubt that both *Aedes aegypti* and *Anopheles annulipes* transmit the myxoma virus by this means and by no other.

Suggestions that myxoma virus (or its close relative fibroma virus) does multiply in mosquitoes have been made by Jacotot *et al.* (1954) and by Kilham & Dalmat (1955). The assertion made by Jacotot *et al.* rests entirely upon the interval of time (21 days in their experiment) which may occur between the acquisition feed and a subsequent infective feed. Our results provide ample grounds for believing that such occasional positive results are due only to persistence of virus on the contaminated mouthparts, and do not constitute valid evidence for multiplication in the mosquito. Kilham & Dalmat provided somewhat more suggestive evidence, but the analysis provided in Table 5 of this paper suggests that the apparent increase in virus content of mosquito suspensions after several weeks was almost certainly due to chance variations in the virus load of infected mosquitoes plus the irregularities introduced by the methods of grinding and titration employed.

It was not possible to demonstrate the location of virus on the mouthparts of the mosquito. The portions of the mouthparts which are inserted on biting are the maxillae, mandibles, labrum-epipharynx and hypopharynx. Of these the maxillae offer the greatest opportunity for the lodgement of virus particles in a position from which they could be dislodged on subsequent probing. The anatomy of the maxillary stylets of several species of mosquitoes is illustrated in Pl. 6. If virus particles adhere to the stylets they are maintained in an environment in which they are continually bathed in fluid. The survival time of virus particles in such a situation is much the same as in a suspension of infected tissues in fluid containing a high concentration of protein (compare 20% loss per day in Table 12 and half

lifetime of 31 hr. at 27° C., Text-fig. 2). It is clear from Text-fig. 2 that if a mosquito acquires a heavy virus load, as some insects undoubtedly do, occasional positive results may be expected after long intervals of time, especially with hibernating mosquitoes, which are maintained at a low temperature.

Virus was found in the head of the mosquito as well as on the stylets. The action of the powerful pharyngeal dilator muscles must result in cell constituents, sometimes including virus particles, being withdrawn from cells penetrated by the stylets. Some virus particles may remain in the gut, particularly in the buccopharyngeal armature. It is possible, also, that virus is wiped off some part of the proboscis and deposited on the head during the toilet performed with the forelegs after probing or feeding. There is no reason to believe, however, that virus contained in the head could cause infection on subsequent feeding. As regurgitation does not occur it is hard to visualize a mechanism whereby virus lodged in the buccopharyngeal armature could be injected into a susceptible host.

Several unsuccessful attempts were made to render mosquitoes infectious by allowing them to feed on a cotton-wool pad soaked in rabbit blood containing virus, but the titres of virus never exceeded $10^{6\cdot3}$ rabbit-infectious doses per ml., and often fell well below this by the end of the feeding exposure. Correlations between the skin titre of virus and the likelihood of mosquitoes acquiring infection (Fenner *et al.* 1956) showed that infections were rare with titres lower than 10^7 rabbit-infectious doses per g., and as this titre was never reached and maintained throughout an experiment involving feeding on cotton-wool pads, the observed failures were not unexpected and no conclusions can be drawn concerning the uptake of virus during feeding on watery suspensions.

It is probable that all the insect-borne pox viruses of animals are transmitted mechanically. The work of Brody (1936) on fowlpox, of Shope (1940) on swinepox and of Philip (1942) on rabbit fibroma suggests that in each case transmission is mechanical and that the mosquito, louse or reduviid vectors act merely as 'mobile pins'. Further, it is probable that mechanical transmission by insects is an important mode of transmission of other viruses which produce viruliferous skin lesions, such as sheep pox and goat pox (Bennett, Horgan & Haseeb, 1944), rabbit papilloma, contagious ecthyma of sheep, and perhaps even cowpox and smallpox.

If we accept the fact that transmission is mechanical the statistical analysis of the experiment in which a mosquito is induced to probe a series of sites (set out in the appendix to this paper), permits the realization of the second aim of this paper, namely, to establish a quantitative measure of insect transmission of myxoma virus. The epidemiological consequences of the application of this measure to several strains of myxoma virus and to different species of insect will be discussed in the following paper. Here it is sufficient to note that the method permits the calculation of the median minimum number of infectious virus particles acquired by a mosquito. With the Lausanne strain, under the conditions of the experiment reported here, this approximates eight virus particles. It should be stressed that the maximum may be many times this figure, and that a considerable percentage of mosquitoes feeding on a lesion acquire no virus load or very little. The explanation for both observations is the very irregular distribution of

virus in the skin lesions, shown in sections stained with fluorescent antibody. Some 12% of the number of infectious particles acquired are left in the skin each time a mosquito probes, and roughly 20% of the infectious particles are lost in other ways each day following the acquisition feed.

SUMMARY

Proof of the existence of multiplication of myxoma virus in mosquitoes has been sought by a variety of experiments with *Aedes aegypti* and *Anopheles annulipes*. All were completely negative. All features of transmission are compatible with a purely mechanical and none is compatible with a 'biological' mechanism.

In mechanical transmission important features of the infected animal host are the number and accessibility of viruliferous skin lesions, and the location and concentration of virus in these lesions.

By inducing mosquitoes to probe through infectious skin lesions and subsequently permitting them to make many successive probes on marked skin sites on the backs of susceptible rabbits, it has been possible to obtain quantitative information on the median minimum virus load of probing mosquitoes, and the rates of loss due to probing and the passage of time.

A preparation of myxoma virus suspended in normal rabbit serum had a half-lifetime of 11 days at 4° C., 5 days at 18–20° C., and 31 hr. at 27–28° C. Apart from losses due to probing (about 12% of the virus load per probe) viable virus on the proboscis of the mosquito probably disappears at about the same rates.

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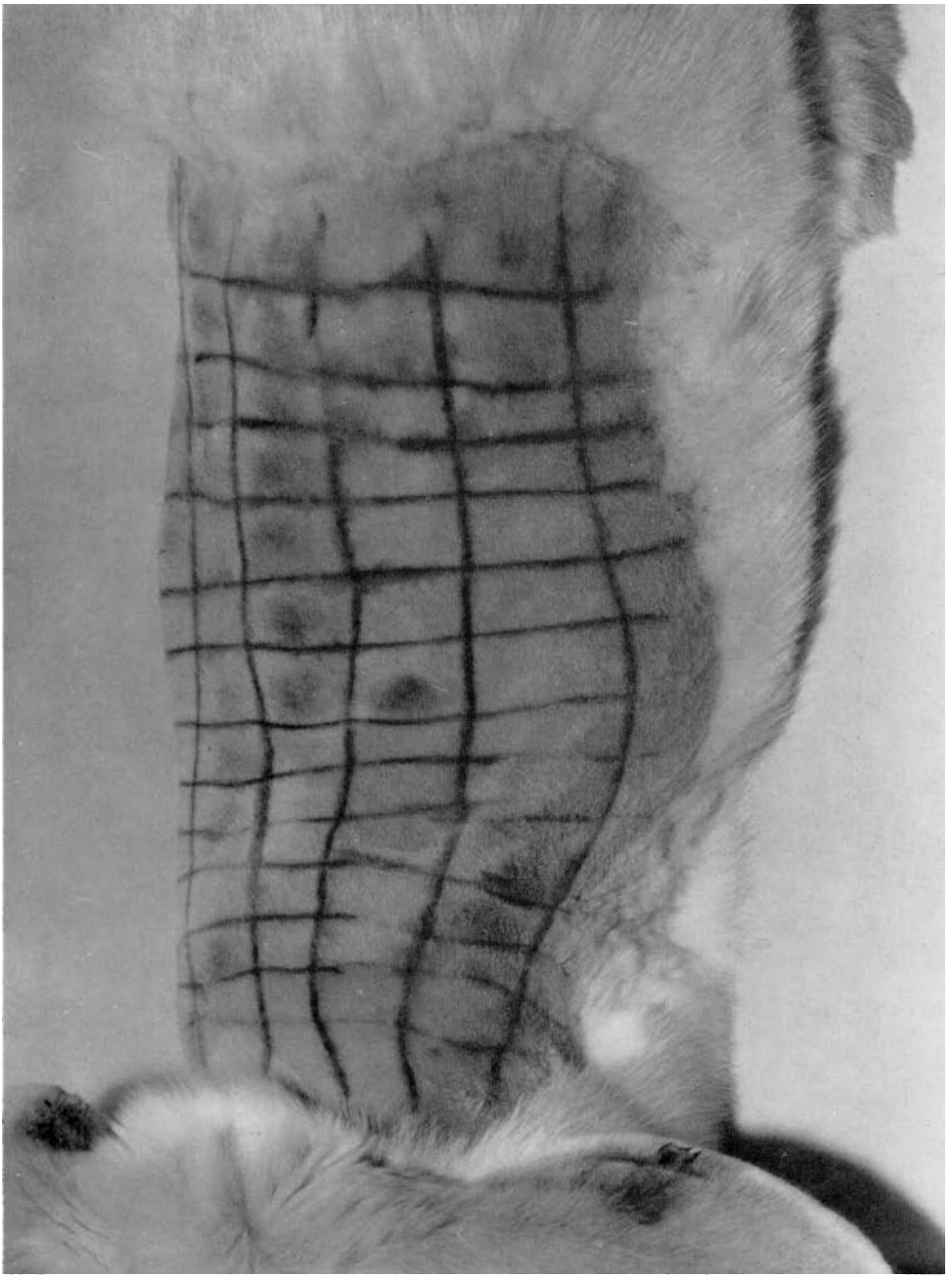

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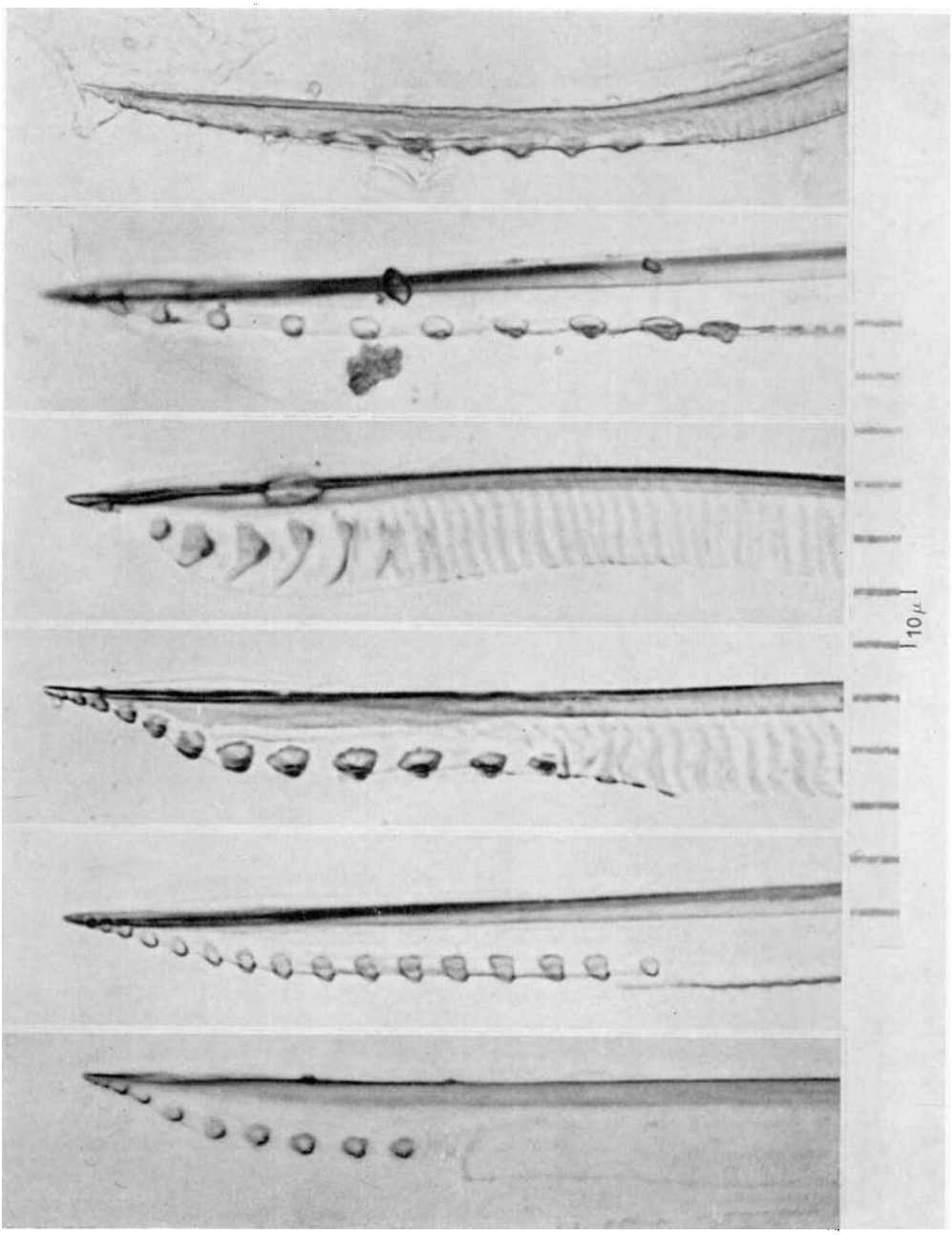

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EXPLANATION OF PLATES

PLATE 5

The appearance of the shaved back of a white rabbit infected with myxomatosis four days earlier by the probes of mosquitoes in the marked skin sites. Primary lesions occur at the sites of the infective bites.

PLATE 6

Photomicrograph of the maxillary stylets of several species of mosquito found in Australia. From left to right: *Aedes aegypti*, *Aedes alboannulatus*, *Aedes vigilax*, *Mansonia uniformis*, *Culex annulirostris*, and *Anopheles annulipes*.

(*MS. received for publication* 28. XI. 55)

APPENDIX

ANALYSIS OF MOSQUITO PROBE DATA

BY G. A. MCINTYRE

The method of analysis of data consisting of the successes and failures of successive probes by mosquitoes can be illustrated by the data of an experiment using the Lausanne strain. The object of the experiment was to determine the rate of loss of virus with repeated probing and with lapse of time from the infective feed.

A particular mosquito after an interval of either 0, 48, 72 or 96 hr. after an infective feed was allowed to probe a rabbit at a recorded position but was removed before settling to a blood meal. This probing was repeated up to twenty times if the mosquito would co-operate for so long. For most of the insects the records are complete to the nineteenth probe, and for purposes of this discussion only those mosquitoes which made at least nineteen probes will be included in the final analysis.

Let us consider a model to serve as a basis for analysis. There is potentially a variable initial load for mosquitoes and a variable rate of wipe-off. If the proportion lost per probe is constant and only a small but constant proportion of the particles wiped off will cause an infection, then the position is analogous to a series of dilution assays of unknown concentrations with unknown rates of dilution and with one replicate at each of only nineteen dilutions irrespective of the initial concentration. For progress, the first simplifying assumption to be made is that the rate of loss per probe, i.e. the dilution rate, is uniform over all mosquitoes.

For an initial survey of the data, possible mosquito variation in initial load was ignored, and all data, including records of mosquitoes which did not complete nineteen probes, were grouped to give the proportion of misses (p) at each successive probe for each of the four periods before testing. If we take λ, the mean number of infections per probe, for successive probes as λ_0, $\lambda_0 R$, $\lambda_0 R^2$, etc., then $\log \lambda$, and therefore $\log(-\log p)$, decreases linearly with probe number. The constants of the line may be determined by the method of maximum likelihood for which Mather (1949) has supplied the necessary tables. The slope of the line is $\log R$ or $R = e^b$, where R is the fraction retained per probe. This preliminary analysis

gave the following slopes and residual sum of squares of deviates about the regression line:

Interval (hr.)	Regression slope	Residual sum of squares	Degrees of freedom
0	−0·1121	11·91	17
48	−0·1384	6·75	17
72	−0·1216	14·93	17
96	−0·1271	9·62	17

The weighted slope is −0·1242 with a corresponding R of 0·8832. The regression slopes are not significantly different and give support to a reasonable hypothesis that the rate of wipe-off per probe is the same independently of the period which elapses from the infective feed. The residual sum of squares is suspiciously less than would be expected from the degrees of freedom.

However, it was evident from inspection of the data that the variation in number of successful probes for different mosquitoes was greatly in excess of pure chance, assuming that their initial loads and rates of wipe-off were the same. Under these assumptions the probability, p, of a failure at a particular probe is given by the proportion of failures at this probe.

An improved P is given by reading back from the regression line fitted to $\log(-\log p)$. The variance of a failure for an individual mosquito at probe k is P_kQ_k and the variance of the total number of failures is $\sum_1^{19} P_kQ_k$, assuming independence of failure in successive probings. With high infectivity of the virus particles there will be a negative correlation between successive probes so that $\sum_1^{19} P_kQ_k$ will overestimate the expected variance of total failures. $\sum_1^{19} P_kQ_k$ is closely approximated by $\sum_1^{19} p_kq_k$, where p_k is the observed proportion of failures at the kth probe. The variances of the total number of failures, out of nineteen probes, among individual mosquitoes are compared (in the following table) with the maximum variances expected on the above assumptions:

Interval from infective feed	0 hr.	48 hr.	72 hr.	96 hr.
$\Sigma_{p_kq_k}$	3·72	3·03	2·52	2·64
Variance of total failures	31·43	15·91	8·76	12·00
Variance ratio	8·45	5·25	3·48	4·54
Probability	<0·001	<0·001	<0·001	<0·001

There is therefore no doubt that the mosquitoes do vary among themselves in their capacity to cause infection, and the simplest hypothesis is that this is due to variation in initial load.

The next stage in the analysis was the estimation of rate of wipe-off eliminating heterogeneity in initial load as far as possible. Mosquitoes were grouped into four classes, irrespective of time since the infective feed, according to their total number of failures. The proportions of failures at successive probes in these four classes were analysed for slope and residue by the Mather procedure. In excluding

mosquitoes with totals less than five and greater than seventeen a bias may have been introduced into the estimate of the slope:

Class	Range of total failures	Regression slope	Residual sum of squares	Degrees of freedom
A	17–15	−0·1790	19·98	17
B	14–12	−0·1139	19·63	17
C	11–9	−0·1129	24·19	17
D	7–5	−0·1343	12·31	17
		−0·1291	76·11	68

The regression slopes are not significantly different, and the residual sums of squares conform satisfactorily with expectation. The fraction retained per probe, calculated from the weighted mean regression slope, is $e^{-0\cdot1291}$ or 0·8789.

It can be shown that the effect of heterogeneity is to decrease the slope and to underestimate the weights for fitting the regression line by a factor of $(1-\sigma_p^2/\overline{pq})$, where σ_p^2 is the variance between mosquitoes in the probability of failure at a particular probe and $\overline{p}$ is the corresponding mean probability. The effect of the heterogeneity decreases as the heterogeneity lessens, and the only issue is whether with the grouping of the data in this trial it is of any consequence. If an approximate estimate of λ for a particular mosquito be taken as that value for which $\sum_1^{19}\exp(-\lambda R^{k-1})$ equals the total number of failures where $R=0\cdot8789$, then for class D, for example, the range of λ is estimated as 3·25–4·75. The actual range of λ's generating the total failures in this class is of course uncertain. With two mixed populations of equal size with $\lambda=3\cdot25$ and 4·75, the fitted line to the composite distribution has a slope of −0·1279 and R is 0·8800. It can therefore be accepted that heterogeneity effects on the value of R are negligible.

The model so far considered is one in which the probability of loss of a particle at each probe is the same and the proportion of removed particles which cause an infection is small and is constant from probe to probe. This is one extremity of the range of possibilities with regard to infectivity. By starting from the hypothesis that every particle which is lost produces an infection, it can be shown that the probability of no particles being lost at the kth probe is $(1-p_1q_1^{k-1})^N$, where N is the initial number of particles and p_1 is the probability of loss of each particle per probe. If the probability that a wiped off particle will cause an infection is p_2 then the probability of no infection at the kth probe is $(1-p_2\,p_1\,q_1^{k-1})^N$. With $p_2\,p_1$ small this is virtually $\exp(-\lambda_0\,q_1^{k-1})$, where λ_0 is $p_2\,p_1\,N$. This is the form assumed in the Mather model with q_1 equal to R.

To examine the effect of high infectivity on the estimate of R or q_1, numerical examples were taken with $p_2=1$, $q_1=0\cdot8789$, $N=2$, 10. The line fitted to

$$(1-0\cdot1211\times0\cdot8789^{k-1})^N$$

by the Mather procedure with $k=1$ to 19 gave an estimated value of R or q_1 of 0·8758 and 0·8760 respectively, so that with complete infectivity the rate of retention is slightly underestimated. The effect of variation in N is also small since the variation only influences the weights in fitting the regression line. With $p_2=0\cdot5$ and $N=2$, R is estimated as 0·8776, so that even at this high rate of infection there

is virtually no disturbance to the Mather model. The curvature of the regression line associated with the departure from the strict dilution relation is negligible.

The next point to be considered is an estimation of the initial number of infections per probe. If the rate of retention per probe for all mosquitoes is taken as 0·8789, then an estimate of the value of λ_0 for an individual mosquito can be obtained from its record of successes and failures using a maximum-likelihood procedure for dilution assays with known dilution factor. It is more economical in computation and causes little loss of efficiency to take as the estimated λ_0 that value for which $\sum_{1}^{19} \exp(-\lambda R^{k-1})$ is equal to the number of failures. The curve relating λ to the expected number of failures is J-shaped with an expectation of nineteen failures with λ zero and zero failures with λ at infinity. The process of reading back values of λ_0 from total number of failures results in an estimate whose variance is greater than the variance of the true λ_0 values for a group of mosquitoes.

It is of some importance to have a measure of central tendency of the true λ_0 value for a group of mosquitoes which will not be seriously biased. Because of the curvilinear relation between the expected number of failures and λ_0, reading back from the mean of failures will give a biased estimate of mean λ_0. With small dispersion in the number of failures for given λ_0, the median of the number of failures and the expected number of failures corresponding to the median λ_0 of the population would be virtually identical but with substantial dispersion this may not be so.

A limited exploration of the point was made arithmetically. So far as one can judge from the distribution of λ_0 for mosquitoes, obtained by reading back from the number of failures, the true frequency distribution of λ_0 is strongly skew positive for low median λ_0 but approaches symmetry with increasing median λ_0. Two arbitrary probability distributions of true λ_0 values were examined.

(*a*) $Z = e^{-x}$ from $x = 0{\cdot}0$ to 3·5, $Z = 0{\cdot}0830 - 0{\cdot}0151x$ from 3·5 to 5·5. This was rescaled to give similar distributions with upper limits of 8·5 and $5{\cdot}5^2/8{\cdot}5$ or 3·559.

(*b*) A symmetrical distribution with relative frequencies in intervals centred at $\lambda_0 = 1$ to $\lambda_0 = 8$ of 0·03, 0·10, 0·17, 0·20, 0·20, 0·17, 0·10, 0·03.

The relative frequencies of 0–10 failures from ten probes with $R = 0{\cdot}8789$ were computed for selected values of λ from 1/16 to 8 by expanding the product $\prod_{s=1}^{s=10} (p_s + q_s)$, where $p_s = \exp(-\lambda R^{s-1})$. The frequencies for the continuous skew distribution with the selected values as mid-points of successive intervals were determined. The proportion of mosquitoes with ten failures was then computed by summing for λ_0 the products of the proportion of mosquitoes centred about a λ_0 value by the expected fraction of insects with this λ_0 which will have ten failures. Similarly for 9, 8, 7, etc., failures. The median of the probability distribution of failures generated in this way was calculated on the assumption that the proportions represent areas with the centres of the unit intervals at 10, 9, 8, ..., 0. A second degree parabola was fitted to three adjacent intervals containing the median so as to give the same areas under the curve as the frequencies. The median was estimated as that limit to the integral under the curve for which the total area below the limit is 0·5. The value of λ_0 corresponding to this median was computed

by inverse interpolation. The following results were obtained. The estimate of the population mean is included for comparison:

	Population median	Estimated median	Population mean	Estimated mean
Skew distribution:				
Upper limit 8·500	1·071	1·086	1·530	1·106
5·500	0·693	0·687	0·990	0·795
3·559	0·449	0·438	0·640	0·554
Symmetrical distribution	4·500	4·522	4·500	3·789

This numerical examination was very limited and approximate, but it did indicate that the bias involved in taking the population median λ as the value read back from the sample median failure was small. It would appear from the asymmetrical series that if the rate of loss of virus with time is estimated from the medians the loss will be slightly overestimated as a consequence of the procedure. There is the additional complication that the ratios of medians of populations will not be the same as the ratio of the means if the form of the distribution changes as a consequence of chance variation between mosquitoes in the proportion of virus lost.

For the Lausanne data, for insects with nineteen probes, the following values were obtained:

Interval from infective feed (hr.)	Median number of failures	Equivalent λ_0	$\log_e \lambda_0$
0	13·00	1·05	0·049
48	14·25	0·78	−0·249
72	16·12	0·43	−0·844
96	16·50	0·36	−1·022

A line fitted by least squares to $\log \lambda_0$ with equal weight for all points gave a slope of $-0{\cdot}282$ per day or a rate of survival per day of 0·75. Including all the data the estimate of survival per day is 0·80.

Finally, in the event of every particle removed by probing causing a lesion, one can make an estimate of the median initial number of particles present. Reverting to a previous example, the regression line fitted to the expected proportion of failures at successive probings for $R = 0{\cdot}8789$ and $N = 2$ has a slope of $-0{\cdot}1326$, an estimated R of 0·8758 and an estimated value from the line for $\log \lambda_0$ of $-1{\cdot}3595$ or $\lambda_0 = 0{\cdot}2568$.

If $e^{-\lambda_0}$ is equated to R^N, the expected proportion of failures at the first probe, $N = \lambda_0 / -\log R = \lambda_0 / -b = 1{\cdot}937$.

If λ_0 is equated to $N(1-R)$ then $N = 2{\cdot}0676$. The correct value of N lies almost midway between these two estimates. This approximate relation applies over a wide range of values of R and N which have been examined. Consequently an estimate of the minimum number of particles corresponding to an estimated λ_0 is $(\frac{1}{2}\lambda_0)\left(\frac{1}{1-R} - \frac{1}{b}\right)$. For these data an estimate of the median λ_0 using all information is 0·98. With a regression slope of 0·1291 and an R of 0·8789, the estimate of the corresponding minimum median number of infective particles is 7·8.

REFERENCE

MATHER, K. (1949). *Biometrics*, **5**, 127.