A GROUP OF VIRUSES ISOLATED FROM NATURALLY INFECTED MOSQUITOES COLLECTED IN THE MURRAY VALLEY AREA OF VICTORIA AND NEW SOUTH WALES

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(With Plates 16 and 17)

The isolation of the virus of Murray Valley encephalitis during the outbreak of this disease in 1951 (French, 1952), and a study of the epidemiology (Anderson, Donnelley, Stevenson, Caldwell & Eagle, 1952) led to a search the following year for the possible arthropod vector. The collection of some 43,000 mosquitoes and the examination of about 18,000 of them for Murray Valley encephalitis virus without finding this organism has been described elsewhere (Reeves, French, Marks & Kent, 1954).

The present paper describes the isolation and some properties of six viruses isolated from pools of *Culex fatigans* and also a seventh isolated from a pool of *C. annulirostris* mosquitoes collected in the Mildura area of the Murray Valley. Each virus was isolated on the chorio-allantoic membrane of developing chick embryos in which situation it produced pocks. Five of the isolated viruses appear to be members of the bird pox group, while the other two, differing in the type of lesion they produce on the chorio-allantoic membrane, appear to be previously undescribed mosquito-borne viruses.

ISOLATION OF THE VIRUSES

The technique used in the collection, storage and preparation of the mosquitoes for inoculation into chick embryos has been described previously in some detail (Reeves *et al.* 1954). Briefly the essential points were: (1) the mosquitoes were mostly collected with sucking tubes from a variety of resting places by day and by night, occasionally by attraction to human or animal bait; (2) mosquitoes were held alive for 24 hr. after collection to permit digestion of freshly taken blood meals and were then identified, sealed in tubes and held frozen on solid CO_2 until ready for examination for virus; (3) suitable pools of mosquitoes were ground with sterile alundum to a moist paste and then taken up in sterile rabbit serum saline, which was centrifuged and the supernatant mixed with penicillin and streptomycin

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before inoculation into chick embryos. Six chick embryos 11 days old were inoculated with 0·1 ml. of the mosquito suspension on the chorio-allantoic membrane by the method of Beveridge & Burnet (1946). The inoculated embryos were incubated for 3 days at 35° C. and the chorio-allantoic membranes then examined for lesions.

The first virus isolated, CF–1, was from a pool of *C. fatigans*. The inoculated chorio-allantoic membranes examined on the third day showed oedema only, but on passaging these membranes to further chick embryos and examining after 2 days, between 5 and 20 pocks about 1 mm. or less in diameter were seen on each membrane.

A pool of 50 *C. fatigans* provided the virus CF–2. After 3 days' incubation the inoculated chorio-allantoic membranes were found to have between two and five dense, white, raised lesions between 2 and 4 mm. in diameter. On passage many pocks were seen tending to produce an opaque thickened oedematous membrane after 3 to 6 days' incubation at 35° C.

The virus CF–3 was isolated from a pool of 50 *C. fatigans*. Four of the membranes inoculated each showed two large, white, dense pocks with an area of oedema about the lesions. The other two membranes were oedematous but no pocks were seen. On passage to further chick embryos all inoculated membranes showed well-developed pocks in 3 days.

From a pool of 59 *C. fatigans* the virus CF–4 was isolated. This lot of mosquitoes was the most heavily infected pool examined and pock counts on the directly inoculated chorio-allantoic membranes showed 17, 16, 4, 22, 5 and 12 pocks per membrane. The lesions were similar to those produced by CF–3 virus and both differed from CF–2 in being slightly smaller (1·5–3 mm. in diameter) and having a surrounding ring of oedema about the opaque pock.

A pool of 61 *C. fatigans* contained CF–5 virus. Suspicious lesions were seen on two of the six membranes inoculated primarily, but undoubted pocks were not recorded. On passage, however, all membranes were found to have between 200 and 300 pocks similar to those produced by CF–1 virus.

Following the inoculation of embryos with another pool of 74 *C. fatigans* all membranes were found to be oedematous but no pocks were seen. These membranes were passaged and areas of translucent oedema about 2–4 mm. in diameter were found after 3 days' incubation. These membranes were passaged to further chick embryos and one, examined after 3 days' incubation, was found to be grossly oedematous but otherwise normal. The remaining three embryos were allowed to incubate for a further 3 days. On examination of these membranes 6 days after inoculation two embryos were found to have died just before examination and showed the grossly thickened opaque membrane with tortuous blood vessels seen in confluent lesions of CF–2 and fowl-pox viruses. One embryo was still alive with confluent membrane lesions, in the surface of which vesicles could be seen. Overlying the membrane was about 1·0 ml. of slightly turbid fluid. It seems certain that the oedematous translucent lesions seen in the first passage membranes after 3 days' incubation were specific pocks, and that these would have developed into the classical fowl-pox-like lesions if incubation had been allowed to proceed for 5 days instead of 3 days. This virus has been designated CF–6 in the series.

Another species *C. annulirostris*, provided the virus CA–1 from a pool of fifty mosquitoes. After 3 days' incubation the membranes inoculated with the mosquito suspension showed between one and five oedematous plaques. By passaging these and allowing incubation to proceed for 5 days, a semi-confluent lesion was found on all membranes.

All attempts to isolate virus from mosquitoes were carried out in a room away from work on other viruses. Hands were carefully washed with soap and hot water after handling each pool of mosquitoes. With those isolations in which undoubted virus lesions were not seen following primary inoculation of chick embryos, re-isolation from an aliquot of the mosquito suspension store in sealed ampoules in solid CO_2 was undertaken. This was successful in each case, viz. CF–1, CF–5, CF–6 and CA–1. As soon as undoubted virus lesions were observed on the membranes further work on them was done in a different room, away from that in which the mosquitoes were being examined. These precautions were aimed at preventing laboratory cross-infection of virus.

ENVIRONMENTS FROM WHICH THE VIRUSES WERE ISOLATED

The seven viruses were isolated from mosquitoes obtained in a very wide range of ecological habitats and collected by several techniques. These environments and techniques are described separately for each virus isolation because more than one virus is represented in this group.

The area from which all the mosquitoes collected were obtained has been described in some detail elsewhere (Reeves *et al.* 1954). It was a limited region of north-western Victoria and southern New South Wales centred about the town of Mildura. The area extended westward to Wentworth in New South Wales and southward to Red Cliffs in Victoria. This encompassed an area along the Murray River approximately 30 miles long and extending back several miles from the river on the Victorian side and several miles up the Darling river on the New South Wales side.

Isolation CF–1 was made from a pool of *C. fatigans* collected by aspirator from daytime resting places in hollow stumps on the margins of the Murray River several miles from Merbein. This was a natural river-bottom flood-plain environment with fairly dense eucalyptus cover and inhabited by a variety of wild birds, a few rabbits, and occasional horses and cattle. The closest domestic birds were several miles away. The primary mosquito breeding sites were residual pools left after river flooding.

Isolation CF–2 was made from a pool of *C. fatigans* collected from a domestic habitation located on the bluff above the Murray River flats in the town of Merbein. These specimens were collected by aspirator from daytime resting places in the shade of a verandah, and from a bathroom and laundry room. Conspicuous in the vicinity were domestic fowl and wild birds (primarily English sparrow), cows and the usual domestic pets. The *C. fatigans* probably came from domestic water breeding sources.

Isolations CF–3 and CF–4 were from two collections of *C. fatigans* obtained from a domestic habitation located on the fringe of the Merbein residential area and on

the bluff immediately above the Murray River. The isolations were made from collections of daytime mosquitoes from a bathroom and chicken house respectively. The vertebrate fauna of this habitat represented a mixture of domestic and wild representatives due to the immediate proximity of the river. The mosquitoes possibly came from both domestic and river margin breeding sources.

Isolations CF–5 and CF–6 were from collections of *C. fatigans* made in a domestic habitation on the edge of a billabong. Resting mosquitoes were collected by aspirator from the verandah, bathroom and sheds of these premises. The immediate fauna was of domestic animals such as chickens and cattle; however, infiltration by a variety of wild bird species was continuous. Several immediate sources of *C. fatigans* were noted in watering troughs and a water tank.

Isolation CA–1 was from a pool of *C. annulirostris* collected by aspirator from a cow. This bait cow was tethered in an old channel of the Murray River on the edge of King's Billabong, a large body of water with an extensive marshy margin, a breeding source of mosquitoes. The immediate domestic fauna consisted of a cow, a dog and five chickens. The wild avian fauna in this area was particularly numerous and representative of a large number of species.

PROPERTIES OF THE ISOLATED VIRUSES

As it was isolated, each virus was inoculated into suckling mice intraperitoneally and adult mice intracerebrally. None produced disease in mice of either age.

The appearance of the pocks on the chorio-allantois produced by CF–2 virus was suggestive of fowl-pox virus, and attempts were made to infect laboratory-reared White Leghorn fowls with CF–2, CF–3, and CF–4 viruses. A 10% emulsion of a confluent infected membrane was scratched on to the comb of the fowl and at the same time a few feathers were plucked from the breast and a little of the emulsion injected into the feather follicles. With CF–2 virus some small vesicles developed on the comb and these went on to crust formation. However. the lesions were not as large nor as extensive as those produced by classical fowl-pox virus. The inoculated feather follicles became swollen on the sixth day and remained so for 4–5 days after which they subsided. A biopsy of the lesion of the skin and feather follicles was taken on the sixth day and the tissue titrated for virus on the chorio-allantoic membrane. The titre was found to be 8×10^6 per gram of tissue: the blood, titrated at the same time, contained no detectable virus at a dilution of 1/100. The other two viruses, CF–3 and CF–4, failed to cause any lesion in fowls when inoculated in this way, but neutralizing antibody developed after the inoculations.

CF–1 and CF–5 produced pocks so obviously different from those of CF–2, CF–3, CF–4, CF–6, and CA–1 viruses that they were thought to belong to a different group. Rabbits were inoculated intracutaneously and by scarification of the cornea with CF–1 and CF–5 with negative results and this indicated that they were not strains of myxoma virus which was prevalent in the area where the mosquitoes were captured. These two viruses were also inoculated intramuscularly into pigeons, young chickens and adult fowls without causing any symptoms, and no neutralizing antibody formation could be demonstrated following multiple inoculations into rabbits, pigeons or adult fowls.

All seven viruses were filterable through gradocol membranes of 800 and 600 mμ average pore diameter. The small pock viruses CF–1 and CF–5 filtered through these membranes without loss of titre but the large pock group showed a fall in infectivity of the order of 3 log units on passage through 600 mμ membranes. More detailed gradocol membrane filtration analysis for the determination of the size of these viruses could not be undertaken because other suitable membranes were not available. The experience with the 600 mμ membrane suggests that CF–1 and CF–5 may be smaller than the other five viruses; on the other hand, it is known that the bird pox group of viruses are often difficult to filter through gradocol membranes due to clumping of the virus particles (Burnet & Lush, 1936).

HISTOLOGY OF THE POCKS ON THE CHORIO-ALLANTOIS

The lesions produced by CF–1 and CF–5 viruses on the chorio-allantois of 10- or 11-day-old chick embryos were morphologically and histologically similar. Examined at about 40 hr. after inoculation a typical membrane showed small areas of ectodermal proliferation as shown in Pl. 16, fig. 1. Under higher magnification evidence of cell proliferation shown by swelling and darker basophilic staining of the nuclei was seen at the edge and base of the lesion. Within the lesion most of the epithelial cells were swollen and had prominent nucleoli but no inclusion bodies could be detected. A few eosinophil polymorphonuclear cells (the typical inflammatory cell of the chorio-allantois) were present. Pl. 16, fig. 2, shows a high-power view of one of the lesions seen in Pl. 16, fig. 1. During quantitative work with CF–1 and CF–5 viruses it was observed that prolonging the incubation period of the inoculated embryos to 3 days caused an apparent fall in the number of pocks seen when the embryos were examined after 2 days' incubation. A membrane infected with CF–5 virus was sectioned after 3 days' incubation and examined. About half the lesions were seen to be in the process of healing. Elongated cells could be seen growing in from the edges of the pock to force the necrotic material away from the membrane and again establish the normal continuity of the surface. This sloughing off of the pock probably accounts for the smaller number of lesions found after 3 days' than after 2 days' incubation, as the healed pocks would not be macroscopically visible. Pl. 16, fig. 3, shows a membrane 3 days after inoculation with a lesion in the healing process.

The viruses CF–2, CF–3, CF–4, CF–6, and CA–1 cause lesions on the chorio-allantoic membrane of a different kind from those of CF–1 and CF–5 viruses. On first isolation CF–2 virus produced prominent, whitish, raised lesions after 3 days' incubation. On section these lesions were seen to be a densely packed mass of more or less necrotic epithelial cells with many eosinophils and some round cells. The ectodermal cells at the base of the pock merged into the necrotic mass and showed large nuclei indicating increased proliferative activity. In some of these cells there were cytoplasmic structures suggestive of inclusion bodies and several nuclei showed mitotic figures. Pl. 17, fig. 4, shows a pock produced by the second egg passage of CF–2 virus. The membrane generally can be seen to be grossly thickened and the mesoderm invaded with many inflammatory cells.

The viruses CF–3 and CF–4 produced lesions similar to those of CF–2 virus, but

macroscopically they were not as dense in appearance nor as raised as those caused by CF–2 virus on first isolation. Microscopically they were seen to be areas of proliferation of the ectoderm with little, if any, inflammatory reaction and none of the necrosis found with CF–2 virus. Pl. 17, fig. 5, shows the lesion produced by the second egg passage of CF–3 virus on the chorio-allantois after 4 days' incubation. The membrane is somewhat thickened with some increased cellularity of the mesoderm, but the pock is due entirely to proliferation of the ectodermal cells.

After several chorio-allantoic passages these viruses produced confluent lesions of the membrane best seen after 5–7 days' incubation at 35° C. At this stage the membranes were very oedematous and 3–5 mm. thick. They were densely white in appearance, often with vesicles and some fluid collected on the surface. There were enlarged tortuous blood vessels on the endodermal side of the membrane. Sections from membranes infected with the fifth egg passage of CF–2 virus and incubated for 6 days were made, and Pl. 17, fig. 6, shows a medium-power view of one of these membranes. The enormous proliferation and down-growths of the ectodermal layer can be seen. The inflammatory reaction and necrosis seen on first isolation of this virus (Pl. 17, fig. 4) have gone. Under higher magnification (Pl. 17, fig. 7) most of the cells in the upper layers of the lesion were seen to contain cytoplasmic inclusions somewhat resembling the Bollinger bodies of classical fowl pox and canary pox lesions (Burnet, 1933). In haematoxylin-stained sections the CF–2 inclusion bodies showed a variety of shapes and sizes and were usually slate-coloured and somewhat hyaline in appearance. Sometimes more than one inclusion was present in a cell and in some cases the inclusion body occupied the entire cell, forcing the nucleus into a thin strip at the periphery of the cell.

SEROLOGICAL RELATIONSHIPS OF THE VIRUSES

White Leghorn fowls were reared from eggs incubated in the laboratory and when about 9 months of age were immunized with CF–2, CF–3 and fowl-pox viruses by a series of subcutaneous inoculations with the living virus. Serum samples were obtained before and at intervals after inoculation. These serum samples were stored frozen without preservative at −20° C. until required. Before being used in neutralization tests all sera were inactivated by heating at 56° C. for 30 min.

Virus suspensions were made by emulsifying heavily infected egg membranes in saline and storing these suspensions in hard glass ampoules at −70° C. on dry ice. Some preparations were mixed with equal parts of sterile glycerol and stored at −20° C. This method was satisfactory, but over a period of about 6 months the titre of the stored virus dropped by about 2 log units. The material stored at −70° C. maintained its titre unchanged over this period.

Neutralization tests were carried out by mixing undiluted, inactivated serum with suitable dilutions of the virus, allowing the mixtures to stand at 4° C. for 1 hr. and then inoculating each of six 11-day-old chick embryos on the chorio-allantoic membrane with 0·05 ml. of the mixture. After 6 days' incubation at 35° C. with CF–2, CF–3, CF–4, CF–6, CA–1 and fowl-pox viruses, pock counts were made. It was noted that allowing the dissected membranes to stand for about 20 min. in 10% formol saline made the specific lesions more opaque and consequently more

readily countable. With these viruses, secondary lesions were not troublesome. With CF–1 and CF–5 viruses 10-day-old eggs were used in neutralization tests and post-inoculation incubation was restricted to about 40 hr.

Tables 1–3 show the results in cross-neutralization tests with three different sera and six viruses. With these viruses even the homologous serum leaves a small percentage of surviving virus particles capable of initiating infection on the chorio-allantois. There is thus a big difference between the neutralizing capacity of a serum which allows 10 % of survivors and that of another allowing only 1 % of survivors. If we take the reciprocal of the percentage of survivors and multiply this by 100 we obtain a figure which gives a better idea of the degree of neutralization of the various viruses by the sera.

On the basis of these tests, CF–2, CF–3 and CF–4 seem to be serologically identical and fairly closely related to classical fowl-pox, although fowl-pox antiserum only partly neutralizes them. Such partial neutralization was found by Burnet & Lush (1936) with canary-pox virus and fowl-pox serum. CF–6 was less readily neutralized by sera against CF–2 and CF–3, but is probably a strain of fowl-pox virus because, like the others, it was partly neutralized by fowl-pox antiserum. CA–1, on the other hand, although it shares an antigen with CF–2, CF–3 and CF–4 viruses, was not significantly neutralized by either of two fowl-pox antisera, in tests which, several times repeated, gave essentially the same result each time.

CF–1 and CF–5 viruses were not detectably neutralized by CF–2, CF–3 or fowl-pox antisera, or by a serum aganst infectious laryngo-tracheitis. Antisera to CF–1 and CF–5 made by repeated inoculation of rabbits, pigeons and adult fowls did not neutralize their homologous viruses when tested on the chorio-allantoic membrane. Failure to demonstrate neutralization of CF–1 and CF–5 viruses probably means that they are serologically distinct from the others, or it may mean that, like Murray Valley encephalitis virus, neutralization of these viruses cannot be demonstrated on the chorio-allantoic membrane.

DISCUSSION

The isolation from mosquitoes of the seven viruses described in this paper demonstrates the superiority of the chorio-allantoic membrane of the chick embryo in this type of work. If the more usual technique of intracerebral inoculation of mice had been employed, then these seven viruses would not have been isolated because none of them is able to infect mice.

The serological reactions and histology on the chorio-allantoic membrane of CF–2, CF–3, CF–4, CF–6 and CA–1 viruses leave no doubt that they are members of the bird pox group of viruses. Probably CF–2, CF–3 and CF–4 viruses are identical, while CF–6 seems to be a strain of fowl-pox. The virus CA–1 is closely related to CF–2, CF–3 and CF–4 but antigenically it is quite distinct from fowl-pox virus. The antigenic overlapping between fowl-pox and the CF–2 type viruses is similar to that observed by Burnet & Lush (1936) between Kikuth's canary virus and fowl-pox virus.

The work of Kligler & Ashner (1929) and of Brody (1936) has shown that fowl-pox

Table 1. *Neutralization tests with* CF–2 *immune fowl serum*

Virus	Serum	Dilution of virus 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	$\bar{x}$ S	% survivors
CF–2	F48 normal	—	—	—	9, 13, 20, 23, 17, D	0, 1, 1, 3, 2, 1	—	$16{\cdot}4 \pm 5{\cdot}55 \times 10^4$	4·4
CF–2	F48 CF–2 immune	—	—	10, 5, 14, 3, 4, D	1, 0, 2, 1, 1, 0	—	—	$7{\cdot}2 \pm 4{\cdot}92 \times 10^3$	
CF–3	F48 normal	—	20, 29, 26, 19, 17, 15	3, 2, 2, 1, 2, D	—	—	—	$2{\cdot}0 \pm 0{\cdot}224 \times 10^3$	4·7
CF–3	F48 CF–2 immune	10, 6, 9, 7, 12, 13	1, 1, 1, 1, 0, 1	—	—	—	—	$9{\cdot}5 \pm 2{\cdot}74 \times 10^1$	
CF–4	F48 normal	—	—	29, 35, 42, D, D, D	11, 3, 3, 7, 7, 3	—	—	$5{\cdot}7 \pm 3{\cdot}27 \times 10^4$	3·6
CF–4	F48 CF–2 immune	—	11, 27, 17, 19, 26, 28	1, 2, 6, 0, 4, 1	—	—	—	$21{\cdot}3 \pm 6{\cdot}78 \times 10^2$	
CF–6	F48 normal	—	—	—	22, 38, 42, 36, 38, 29	5, 7, 3, 1, 2, 4	—	$3{\cdot}7 \pm 2{\cdot}16 \times 10^5$	19·5
CF–6	F48 CF–2 immune	—	—	—	3, 8, 8, 9, 6, 9	2, 1, 0, 0, 0, 1	—	$7{\cdot}2 \pm 2{\cdot}32 \times 10^4$	
CA–1	F48 normal	—	—	49, 15, 38, 20, 42, D	1, 2, 7, 9, 0, 3	—	—	$3{\cdot}7 \pm 3{\cdot}56 \times 10^4$	15·4
CA–1	F48 CF–2 immune	—	62, 40, 65, 52, 48, D	6, 5, 10, 4, 4, 5	—	—	—	$5{\cdot}7 \pm 2{\cdot}25 \times 10^3$	
F.P.	F48 normal	—	—	—	38, 42, 27, 22, 33, D	5, 3, 1, 4, 1, 7	—	$3{\cdot}5 \pm 2{\cdot}34 \times 10^5$	12·9
F.P.	F48 CF–2 immune	—	—	—	4, 5, 5, 7, 5, 1	0, 1, 1, 0, 2, 0	—	$4{\cdot}5 \pm 1{\cdot}98 \times 10^4$	

F.P. = Fowl-pox virus.

$\bar{x}$ = mean. S = standard deviation. D = dead embryo. The figures given represent the number of virus particles in 0·05 ml. of a mixture of serum and virus capable of initiating formation of a pock on the chorio-allantois.

Table 2. *Neutralization tests with* CF–3 *immune fowl serum*

Virus	Serum	Dilution of virus 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	$\bar{x}$ S	% survivors
CF–2	F 50 normal	—	—	—	10, 11, 18, 20, 18, 23	3, 2, 0, 1, 4, 0	—	$16{\cdot}7 \pm 5{\cdot}12 \times 10^4$	4·7
CF–2	F 50 CF–3 immune	—	—	3, 7, 9, 11, 9, 8	0, 1, 2, 0, 0, 1	—	—	$7{\cdot}8 \pm 2{\cdot}72 \times 10^3$	
CF–3	F 50 normal	—	21, 32, 24, 20, 16, 18	4, 3, 1, 2, 2, 1	—	—	—	$2{\cdot}17 \pm 1{\cdot}17 \times 10^3$	3·7
CF–3	F 50 CF–3 immune	11, 5, 8, 7, 8, 9	—	—	—	—	—	$8{\cdot}0 \pm 2{\cdot}0 \times 10^1$	
CF–4	F 50 normal	—	—	43, 36, 48, 30, D, D	8, 3, 4, 2, 7, 5	—	—	$4{\cdot}63 \pm 2{\cdot}32 \times 10^4$	2·9
CF–4	F 50 CF–3 immune	—	11, 9, 12, 15, 18, 16	1, 2, 3, 0, 1, D	—	—	—	$13{\cdot}5 \pm 3{\cdot}29 \times 10^2$	
CF–6	F 50 normal	—	—	—	32, 36, 42, 22, 38, D	2, 3, 1, 5, 6, 5	—	$3{\cdot}67 \pm 1{\cdot}81 \times 10^5$	15·8
CF–6	F 50 CF–3 immune	—	—	52, 78, 32, 43, D, D	3, 7, 6, 4, 7, 8	—	—	$5{\cdot}83 \pm 2{\cdot}14 \times 10^4$	
CA–1	F 50 normal	—	—	42, 38, 28, 27, 32, D	3, 2, 5, 4, 4, D	—	—	$3{\cdot}6 \pm 1{\cdot}14 \times 10^4$	18·0
CA–1	F 50 CF–3 immune	—	—	8, 4, 3, 9, 7, 8	0, 1, 0, 2, 0, 0	—	—	$6{\cdot}5 \pm 2{\cdot}39 \times 10^3$	
F.P.	F 50 normal	—	—	—	82, 34, 48, 72, 78, D	7, 6, 2, 8, 10, 4	—	$6{\cdot}17 \pm 2{\cdot}86 \times 10^5$	19·5
F.P.	F 50 CF–3 immune	—	—	—	—	3, 0, 2, 1, 1, 0	—	$12{\cdot}0 \pm 4{\cdot}25 \times 10^4$	

F.P. = Fowl-pox virus.

$\bar{x}$ = mean. S = standard deviation. D = dead embryo. The figures given represent the number of virus particles in 0·05 ml. of a mixture of serum and virus capable of initiating formation of a pock on the chorio-allantois.

Table 3. *Neutralization tests with fowl-pox immune fowl serum*

Virus	Serum	Dilutions of virus 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	$\bar{x}$	S	% survivors
CF–2	F436 *normal*	—	—	—	15, 22, D, 16, 10, 14	3, 3, 1, 1, 1, D	—	15·4	$\pm 4{\cdot}34 \times 10^4$	31·2
CF–2	F436 F.P. immune	—	—	—	3, 2, 13, 3, 6, 2	1, 1, 2, 0, 0, 0	—	4·83	$\pm 4{\cdot}26 \times 10^4$	
CF–3	F436 normal	—	11, 10, 19, 4, 11, D	0, 3, 1, 1, D, D	—	—	—	11·0	$\pm 5{\cdot}34 \times 10^2$	13·2
CF–3	F436 F.P. immune	14, 17, 5, 17, 21, 13	3, 5, 1, 1, 0, D	—	—	—	—	14·5	$\pm 5{\cdot}43 \times 10^1$	
CF–4	F436 normal	—	—	10, 10, 10, 13, 5, 5	5, 0, 0, 0, 0, D	—	—	8·83	$\pm 3{\cdot}19 \times 10^3$	14·1
CF–4	F436 F.P. immune	—	11, 15, 17, 16, 3, D	2, 2, 4, 0, 0, 3	—	—	—	12·4	$\pm 5{\cdot}73 \times 10^2$	
CF–6	F436 normal	—	—	—	—	3, 2, 4, 7, 5, 2	0, 0, 1, 0, 0, D	4·0	$\pm 1{\cdot}79 \times 10^5$	8·5
CF–6	F436 F.P. immune	—	—	49, 32, 23, 27, 33, D	2, 3, 4, 4, 4, D	—	—	3·4	$\pm 0{\cdot}8 \times 10^4$	
CA–1	F436 *normal*	—	—	27, 15, 9, 17, 19, D	9, 2, 0, 1, 4, 0	—	—	17·4	$\pm 3{\cdot}45 \times 10^3$	91·0
CA–1	F436 F.P. immune	—	—	22, 18, 7, 16, 15, 16	5, 2, 0, 2, D, D	—	—	15·8	$\pm 4{\cdot}93 \times 10^3$	
F.P.	F436 normal	—	—	—	—	18, 32, 14, 37, 42, 38,	5, 6, 2, 5, 5, 0	3·8	$\pm 2{\cdot}32 \times 10^6$	1·9
F.P.	F436 F.P. immune	—	—	—	9, 9, 7, 4, 7, 7	2, 0, 0, 0, 0, D	—	7·2	$\pm 1{\cdot}84 \times 10^4$	

F.P. = Fowl-pox virus.

$\bar{x}$ = mean. S = standard deviation. D = dead embryo. The figures given represent the number of virus particles in 0·05 ml. of a mixture of serum and virus capable of initiating formation of a pock on the chorio-allantois.

virus can be carried by mosquitoes. The mosquitoes become infected by biting through a lesion on an infected fowl and so contaminate their mouthparts with the virus. The virus does not multiply in the mosquito and its transmission by this arthropod is purely mechanical. Fenner, Day & Woodroofe (1952) have drawn attention to an essentially similar mechanism with regard to the spread of infectious myxomatosis of rabbits.

The present work provides evidence that a number of agents having the general characteristics of bird pox viruses are carried in nature by *C. fatigans* and occasionally by *C. annulirostris* in that area of the Murray Valley where these mosquitoes were captured. The vertebrate hosts for these viruses may be any of the domestic or wild birds which abound in this area. Kligler & Ashner (1931) succeeded in demonstrating the presence of virus in wild caught *Culex pipiens* mosquitoes by allowing the mosquitoes to bite fowls and also by rubbing the mosquitoes on the scarified comb. The isolations from field mosquitoes reported here are probably the first such isolations to be made using chorio-allantoic inoculated chick embryos, and confirm the idea of mosquito transmission among the bird pox group of viruses.

Table 4. *Neutralization of the viruses by fowl antisera*

	Serum		
Virus	CF–2 (immune)	CF–3 (immune)	Fowl-pox (immune)
CF–2	22·7	21·2	3·2
CF–3	21·2	27·0	7·6
CF–4	27·8	34·5	7·1
CF–6	5·1	6·3	11·8
CA–1	6·5	5·6	1·1
Fowl pox	7·7	5·1	52·6

The figures are obtained by multiplying the reciprocal of the percentage of survivors allowed by a serum by 100.

The two agents CF–1 and CF–5 appear to be unrelated to the five other viruses. They have shorter incubation periods in chick embryos and also produce very small pocks. But histologically they produce the same basic lesion on the chorio-allantois, namely ectodermal proliferation, and moreover they were isolated from the same mosquito species. These two viruses may be the cause of some disease other than a pox disease of birds or animals, but until a host other than the chick embryo is found little can be done to study this aspect further. It might be worth while to attempt to infect some of the commoner wild birds and animals of this area with these two agents to see if some further light can be thrown on their epidemiology.

The ecological environments in which the mosquitoes were captured provide little information concerning the epidemiology of the different viruses. Both CF–1 and CA–1 viruses, which are respectively small and large pock types, were from mosquitoes taken from areas removed from domestic habitation, whereas CF–2, CF–3, CF–4, CF–5 and CF–6 viruses were from mosquitoes collected in close proximity to domestic as well as wild birds and animals.

SUMMARY

By the inoculation of suspensions of mosquitoes on the chorio-allantoic membrane of chick embryos, seven pock-producing viruses have been isolated. The mosquitoes were collected in a small area of the Murray Valley situated in the north-western corner of Victoria and southern New South Wales. Six of the viruses were isolated from pools of *C. fatigans* and one from a pool of *C. annulirostris*. Five of the viruses were found to be members of the bird pox group and related to fowl-pox virus. Two of the viruses differed in the type of pock they produced on the chorio-allantoic membrane and appeared to be serologically unrelated to the other five.

The interest and criticism of Sir Macfarlane Burnet, F.R.S., throughout the conduct of this work is gratefully acknowledged.

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EXPLANATION OF PLATES

PLATE 16

Fig. 1. Pocks on the chorio-allantoic membrane of the chick embryo produced by CF–5 virus 40 hr. after inoculation. H and E., × 120.

Fig. 2. Pock produced by CF–5 virus, showing swollen and proliferated epithelial cells. H. and E., × 350.

Fig. 3. A healing lesion produced by CF–5 virus after 3 days' incubation. H and E., × 144.

PLATE 17

Fig. 4. Dense necrotic lesion produced by CF–2 virus on first isolation. H and E., × 86.

Fig. 5. Lesion produced by CF–3 virus on the chorio-allantoic membrane of the chick embryo after 4 days' incubation. H. and E., × 120.

Fig. 6. Chorio-allantoic membrane heavily infected with CF–2 virus, after 6 days' incubation. H. and E., × 120.

Fig. 7. A higher magnification of the upper layer of proliferated epithelial cells seen in Fig. 6. × 240.

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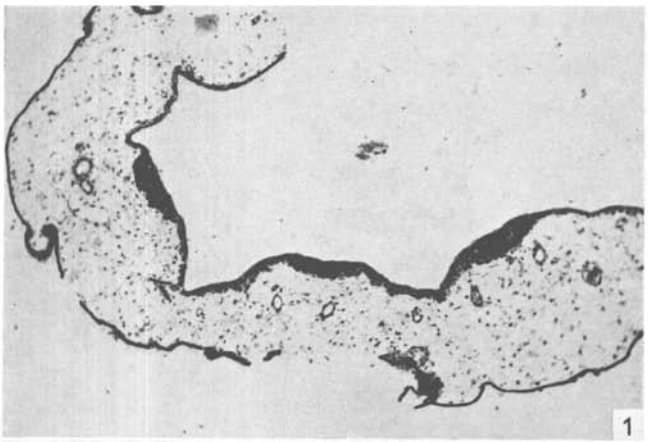

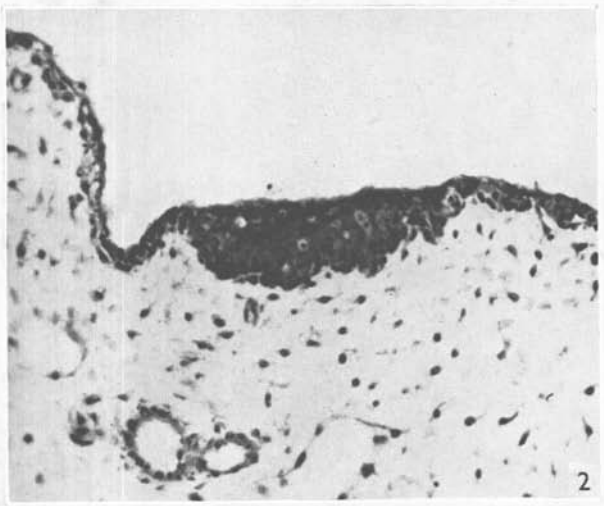

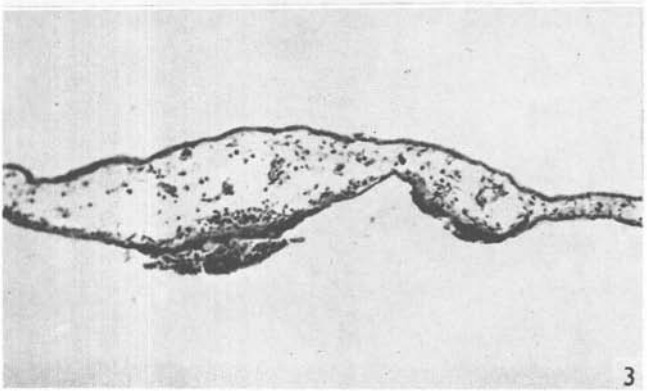

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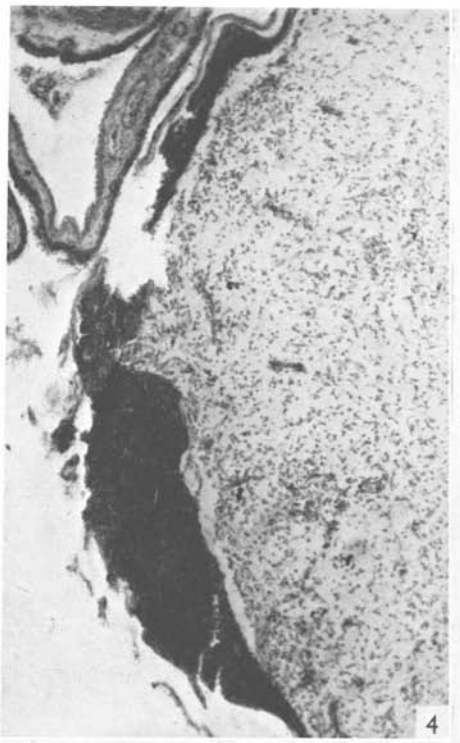

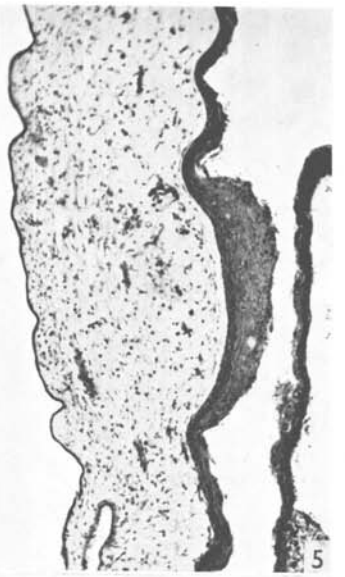

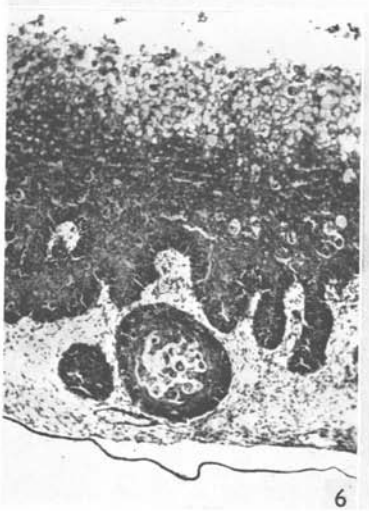

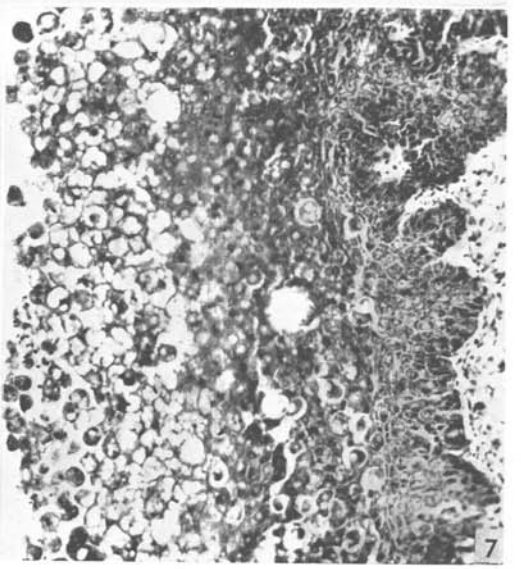

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