cells utilizing a standard vaccinia virus assay[3]. The number of units of interferon in a preparation was the reciprocal of the highest dilution showing approximately 50 per cent plaque reduction as compared with controls.

Chick embryo fibroblast cultures, which had been prepared from 10-day-old eggs, were incubated at 37° C for 4 h with 0·06 μg/ml. or 0·12 μg/ml. of actinomycin *D*, washed with Gey's buffer, and 0·5 ml. of heat inactivated Chikungunya virus or buffer was then added. At the dilution of the Chikungunya virus pool used, the virus plaque forming unit to cell multiplicity was approximately 1 : 1. After incubation at 37° C for 1 or 3 h the cells were again washed and either 2 ml. of buffer was added and the plates incubated at 37° C for 14 h (for interferon yields), or the cells were challenged with 0·5 ml. of an appropriate dilution of NDV, SFV, or Chikungunya virus, and the virus allowed to adsorb at 37° C for 1 h. The cell sheets were then overlayed with 0·9 per cent agar and plaque medium and incubated at 39° C for NDV, at 35° C for Chikungunya, or at 37° C for SFV. In all cases plaques could easily be counted after 24 h of incubation. Final counts were made after 40 h.

Several experiments were performed. The results of a typical single experiment are shown in Table 1. In plates challenged with Chikungunya virus, plaques were completely inhibited by treatment with the heated virus. Pretreatment with 0·12 μg/ml. of actinomycin *D* neither increased the yield of plaques in controls, nor reversed the inhibition of plaques in plates treated with heated virus.

Table 1. Reversal of Viral Interference by Actinomycin *D*

Challenge virus	Actinomycin *D* (μg/ml.)	Interfering virus*	Units of interferon formed	Plaque count at 40 h	Effect found
Chikungunya	0	–	–	22, 24	—
Chikungunya	0	+	20	0, 0	100% inhibition
Chikungunya	0·12	–	–	20, 22	None
Chikungunya	0·12	+	< 4	0, 0	100% inhibition
SFV	0	–	–	215, 225	—
SFV	0	+	20	12, 13	92% inhibition
SFV	0·12	–	–	322, 332	50% enhancement
SFV	0·12	+	< 4	284, 304	35% enhancement
NDV	0	–	—	39, 51	—
NDV	0	+	20	10, 15	67% inhibition
NDV	0·06	–	–	55, 66	35% enhancement
NDV	0·06	+	4	50, 58	20% enhancement

SFV = Semliki forest virus. NDV = Newcastle disease virus.

* –, Heat inactivated virus not added; +, 0·5 ml. of heat inactivated Chikungunya virus added for 1 h for SFV and Chikungunya, and for 3 h for NDV.

In the case of plates challenged with SFV, heated Chikungunya virus reduced the plaque count by 92 per cent as compared to untreated controls. Pre-incubation with 0·12 μg/ml. of actinomycin *D* raised the plaque count by 50 per cent, and by 35 per cent in cells also treated with heated Chikungunya virus, thus completely reversing the interfering action of the latter. In the plates treated with heated Chikungunya virus, the yield of interferon was reduced from 20 units in controls to less than 4 units in plates treated with 0·12 μg/ml. of actinomycin *D*.

In the case of NDV, preliminary experiments had shown that pretreatment with 0·12 μg/ml. of actinomycin *D* decreased the yield of plaques. Also, no viral interference was detected unless the interval between the addition of the heated virus and the NDV challenge was increased to at least 3 h. When a 3-h interval was used, plaque counts were reduced by 67 per cent. When cells were pretreated with actinomycin *D* (0·06 μg/ml.), plaque counts were raised by 35 per cent and by 20 per cent in plates also treated with heated Chikungunya virus. In the case of the latter, however, interferon production was not completely inhibited by 0·06 μg/ml. actinomycin *D*, and 4 units of interferon were found compared with 20 units in the controls.

Since the ease with which the reversal of viral interference by a standard dose of heated virus varied with the different viruses tested, it was of interest to determine the sensitivity of each of the 3 viruses used to a standard preparation of interferon. The inhibitory effect of a 1/20 and a 1/100 dilution of interferon was determined (Table 2). For SFV, a 1/20 dilution inhibited 50 per cent of the plaques, while for Chikungunya virus a 1/100 dilution sufficed; however, for NDV, no significant inhibition was found at a 1/20 dilution.

Table 2. Dilution of Standard Interferon Preparation Inhibiting 50 per cent of Virus Plaques

Test virus	Dilution of interferon reducing plaque yields to 50 per cent of controls
Chikungunya	1/100
Semliki forest virus	1/20
Newcastle disease virus	< 1/20

Listed in order of either increasing sensitivity to interferon (Table 2), or to interference by a standard dose of heated Chikungunya virus (Table 1), the viruses were NDV, SFV, and Chikungunya virus. This is also the order when the ease with which viral interference could be reversed by actinomycin *D* is considered. With NDV, interference was reversed by a concentration of actinomycin *D* which did not completely suppress interferon production. In the case of SFV, a concentration of actinomycin *D* which inhibited the production of interferon reversed the viral interference. For Chikungunya virus, however, interference could not be reversed by a concentration of actinomycin *D* which inhibited interferon production; however, the titres of interferon shown in Table 1 were determined by a vaccinia virus assay system, and Chikungunya virus is 2–3 times more sensitive to the antiviral action of interferon than is vaccinia virus[4]. Thus, a level of interferon which might be undetectable with the assay system used might completely inhibit Chikungunya virus plaque production.

The results are also of interest in that they confirm previously published observations that actinomycin *D* enhances plaque yields of some RNA viruses and depresses production of interferon[1]; however, we were unable to confirm the finding that the yield of Chikungunya virus plaques was increased by actinomycin *D* (ref. 1).

The findings reported here suggest that the effect of actinomycin *D* in reversing viral interference is related to its effect on interferon production. Steroids, which inhibit interferon production[5], have also been shown to reverse viral interference[6]. Both these observations indicate that many examples of viral interference may be very closely related to interferon production.

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Isolation of a Virus resembling Encephalomyocarditis from a Red Squirrel

British red squirrels have suffered from epidemic outbreaks of unidentified disease at various times in the past, sometimes with mortality rates exceeding 90 per cent[1]. At Thetford Chase (Norfolk) sick and dying squirrels were seen recently with symptoms including paralysis of the hind legs. No grey squirrels are known in this area, so it was decided to attempt isolation of virus material from the red squirrel population to initiate a study of virus diseases affecting squirrels in Britain.

An apparently healthy adult female red squirrel was shot in a locality where several affected animals had been

reported. The whole intestine and its contents were homogenized in phosphate buffered saline to make a suspension approximately 20 per cent w/v. Aliquots of this suspension were further treated on an *MSE* ultrasonic disintegrator for 3 periods of 10 sec and centrifuged at 3,000 r.p.m. for 10 min; the supernatant passed through an *HA* grade 'Millipore' filter and the filtrate tested and found negative for bacterial contamination in blood agar plates after incubation overnight at 37° C. The resulting solution was used for inoculations.

Adult mice, 3–4 weeks old, were inoculated intracerebrally, under light ether anæsthesia, with 0·03 ml. of filtrate. They were observed for 14 days and all survived without any apparent signs of infection.

Suckling mice, 24–48 h old, were inoculated intracerebrally with 0·01 ml. inoculum, or in the intrascapular region (fat pad) with 0·03 ml. Of the mice inoculated intracerebrally, 2 of 9 died on the fourth and fifth day and were eaten by the mother, which died on the tenth day. At this time the rest of the litter still appeared healthy.

Of 3 litters of suckling mice inoculated via the fat pad, 4/8, 2/14 and 3/9 died (29 per cent of the total). In all but 2 cases death occurred on the second day.

A suspension made from 3 moribund suckling mice was used to make a further passage. All subsequent suckling mice inoculated intracerebrally or in the fat pad with this first carcass suspension died within 18 h. The first suckling mouse virus stock was made from the skinned and eviscerated carcasses of these infected mice.

L-cell monolayer cultures, in 10 screw-capped insulin bottles at an approximate concentration of 2×10^5 cells per ml. and 2 ml. per bottle, were inoculated with a 1/10 dilution of the gut filtrate. Another 10 bottles were used as controls. At the fifth day all infected cultures showed cytopathic changes and many dead cells could be seen in the fluid.

All 19 suckling and 5 adult mice inoculated intracerebrally with the supernatant of the infected cultures died within 18 h. Controls inoculated with normal *L*-cell extract survived.

The remaining supernatant was frozen and thawed, sonicated, centrifuged as before and inoculated into 12 more bottles. Within 18 h cytopathic changes were observed and by the third day all cells were destroyed. In subsequent *L*-cell passages all cells were destroyed within 18 h.

A further isolation of this agent was obtained after 2 plaques appeared when a 10^{-2} dilution of the original gut filtrate was plated in Krebs cells as described by Sanders *et al.*[2]. Virus stocks were obtained readily from these plaques in agitated suspensions of Krebs cells.

Isolations were attempted using 9-day-old chick embryos; but results proved indecisive.

Virus-like particles of various sizes, the majority being spherical and about 30 mμ in size, were seen in crude preparations with the electron microscope.

The virus was tested for ether sensitivity after shaking for 1 h with an equal volume of ether. Results of intracerebral inoculation into adult mice were:

Non-treated	$10^{-5.5}$ or $1{\cdot}1 \times 10^7$ LD_{50}/ml.
Treated	$10^{-5.6}$ or $1{\cdot}3 \times 10^7$ LD_{50}/ml.

The test was repeated twice with similar results, indicating ether insensitivity. Nucleic acid obtained from crude virus preparations by the conventional phenol method was infective when inoculated intracerebrally into 24-h-old suckling mice. Infectivity was destroyed by RNase but not by DNase, suggesting that this was an RNA virus.

Brain suspensions and carcass suspensions made from infected suckling mice failed to agglutinate red cells of sheep, rabbit, guinea pig, rat, mouse, goose, hamster, or human O group. Tests were made with both ether-treated and untreated virus samples.

Since the virus was of the RNA type, was not ether-sensitive, had spherical particles of the order of 30 mμ in size, and grew readily in mice of all ages, it was assumed to belong to the picornavirus group but not to be poliovirus, coxsackie, or *ECHO* 28-rhinovirus-coryzavirus (ERC) (ref. 3). In spite of failure to agglutinate red cells and to grow in eggs, the readiness with which the virus grew in Krebs cells suggested that it might be a member of the EMC group. Some symptoms observed in infected adult mice had been typical of those produced by EMC.

Neutralization tests were made, using suckling mice inoculated in the fat pad. EMC rat antiserum diluted 1 in 5 reduced by $\geqslant 4$ logs. the titre of suspensions of brain and spinal cord, with a control titre of $10^{-6.5}$ ($1{\cdot}1 \times 10^7$ LD_{50} ml.). This neutralization was of the same order as that against the homologous virus.

Although the virus isolated does not agglutinate red cells it was decided to test for antibodies against standard EMC virus in the serum of wild grey squirrels by *HAI* tests. Taking as a significant level of antibody the ability to inhibit 64 units of HA, 6 out of 9 squirrels tested had antibody against EMC. The validity of the *HAI* test as a means for testing for antibody in populations of grey squirrels was checked in several of the samples by plaque inhibition tests, using Krebs cells. Serum dilutions tested against approximately 100 plaque-forming units confirmed the results of the *HAI* tests.

Groups of 5 adult mice were inoculated intranasally with 0·03 ml. of undiluted first passage carcass suspensions of the virus and a sixth passage brain suspension (3×10^9 plaque-forming units) under light ether anæsthesia. All mice died. Adult mice inoculated orally with the same suspensions all survived the 15-day period of observation, and the pooled blood of these mice showed no antibody to be present. However, a large proportion of mothers which ate infected suckling mice died with symptoms of EMC infection.

One rat, inoculated intramuscularly with $1{\cdot}1 \times 10^6$ suckling mouse LD_{50} first passage carcass suspension for the production of antiserum developed paralysis of the hind legs. Three rats inoculated intranasally with the same inoculum failed to show any outward symptoms of infection, although previous tests for antibody had given negative results.

Three grey squirrels with serum shown to contain no antibody by the *HAI* test were inoculated intranasally with the same inoculum and all developed symptoms. Two showed a steady rise in breathing rate to 166–206 per minute by the sixth day when paralysis of both front and hind legs became apparent. By the eighth day one of these animals also developed a purulent discharge from the eyes. The third squirrel showed no rise in respiratory rate but paralysis of the hind legs was apparent on the sixth day, and death occurred within hours. Post-mortem examination revealed hæmorrhagic patches on the lungs and whitish areas, suggesting necrosis, in the cardiac muscle.

This preliminary work demonstrates the ability of apparently healthy red squirrels to carry a virus capable of infecting grey squirrels. The reverse hypothesis has been offered as an explanation of the decline of red squirrels in the face of an increasing population of grey squirrels.

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