Component fatty acids of the cholesterol esters were quantitated by gas-liquid chromatography.

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Autointerference of Rabies Virus in Chick Embryo Fibroblasts.* (31496)

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In our previous study(1), HEP Flury strain of rabies virus(2) was found capable of forming plaques in primary chick embryo fibroblasts, and the titration of infectivity by this plaque technique has replaced the more laborious baby mouse(2) and one-day egg LD titrations(3). While applying this plaque assay to materials containing a high concentration of virus, we noticed that monolayers receiving an undiluted seed virus suspension formed no plaques. Our investigation of the nature of this phenomenon led to the finding that interferon production was responsible for it. These results are reported here.

Materials and methods. Diluent. The virus diluent was yolk-saline(1), which was 0.01 M phosphate buffered saline of pH 7.2 containing fresh egg-yolk at 0.1%. Just prior to use, penicillin and streptomycin were added at 500 μ/ml and 100 γ/ml, respectively.

Viruses. Standard 7-day egg passage of HEP Flury strain of rabies virus(2) was used at the 259th to 263rd passage levels. Brains of infected embryos were emulsified to make a 20% emulsion in yolk-saline, to serve as seed virus. In one case, the same strain serially passaged in chick embryo fibroblasts(4) was tested; a pool of culture fluids constituted a seed. Small plaque mutant of Western equine encephalitis (WEE) virus(5) and Sindbis virus were at 15th and 51st chick embryo cell passages, respectively, when used. Dairen I strain of vaccinia virus was received from the National Institute of Health of Japan as

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an infected chorioallantoic membrane, and passaged here in HeLa cells; when used in the present experiments, it was at the 11th to 13th HeLa cell passage levels.

Monolayer preparation. Nine-day chick embryos were trypsinized as stated earlier(6), suspended in growth medium at a concentration of 2×10^6 cells/ml, dispensed into 60-mm dishes in 5-ml amounts, and incubated at 37°C. The growth medium consisted of YLE (Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate, 0.1% yeast extract and antibiotics) supplemented with 10% inactivated calf serum and 0.0025 M Tris buffer of pH 7.2. Confluent cell sheets formed in 24 hours and were ready for use.

Plaque assay of rabies virus. The original method(1) was modified as follows. The above monolayers were washed once with phosphate buffered saline (PBS)(7), and virus was inoculated into 3 dishes per dilution in 0.05-ml amounts. Adsorption of virus was allowed to take place at 37°C for one hour, after which 4 ml per dish of an overlay medium was added; the overlay was phenol red-free YLE containing 2% calf serum, 0.022% $NaHCO_3$, 0.0025 M Tris buffer of pH 8.6 and 1% Noble agar (Difco). The dishes were then placed in a humidified 35°C incubator, and 5 days later a second overlay was added, which consisted of 2 ml per dish of 1% agar solution containing 1:10,000 neutral red. The dishes were then transferred to a 20°C incubator, and plaques counted on the next day. PFU means plaque-forming units. For assay of the chick embryo cell passage virus, DEAE-dextran was incorporated into the overlay medium at 0.001%.

Plaque assays of vaccinia, WEE and Sindbis viruses. The method was similar to the above, except that the overlay medium contained 0.11% $NaHCO_3$ with no Tris buffer, and incubation of overlaid dishes was done in a 5% CO_2-air incubator at 37°C. The second overlay was added 3 days following the inoculation.

Bottle culture of rabies virus. Square bottles, $10 \times 5 \times 5$ cm, were seeded each with 1×10^7 chick embryo cells suspended in 10 ml growth medium, rubber-stoppered and incubated at 37°C for 24 hours. Then the cell sheet was washed once with PBS and infected with virus, using an inoculum dose of 1.0 ml. After 2 hours' incubation at 37°C, the cells were washed 3 times with PBS and each bottle received 10 ml of a maintenance medium, which contained 2% calf serum. Further incubation was made at 35°C. Preliminary tests showed higher yields of virus in the fluid than in the cells.

Multiplicity of infection (moi). At the time of virus inoculation, one replicate culture was used for determination of cell number by dispersion with 0.02% EDTA-PBS containing 0.05% trypsin, and the moi was expressed as PFU per cell.

Assay for interferon activity. Pooled culture fluid was dialyzed in the cold against 0.01 M KCl-HCl buffer at pH 2 for 24 hours with a constant agitation by a magnetic stirrer, and then against 0.01 M phosphate buffered saline at pH 7.2 for another day. The resulting fluid was passed through filter paper and stored at −20°C for less than one week, until use. *Quick dialysis* was perfomed by inserting a test tube into a tubing containing the fluid and changing the outer fluid twice at hourly intervals for each buffer. For assay, a test material was diluted with maintenance medium in serial 4-fold steps and added in 2-ml amounts to 3 monolayer dishes per dilution. Control dishes were given the same amount of maintenance medium. The dishes were incubated at 35°C for 24 hours. Then the fluid was aspirated, and all dishes were inoculated with an equal dose of about 100 PFU of vaccinia virus. Plaque assay of vaccinia virus was done as stated above, and the interferon activity was expressed as the highest dilution of the test material initially added which reduced the plaque number to less than half that of control.

Antiserum. This was the same lot as used previously(1), which had been prepared by immunizing a rabbit with mouse brains infected with CVS strain of rabies virus. Its titer was 1:2,600 against 200 mouse LD of the homologous virus.

Results. Autointerference of HEP Flury virus as seen in plaque dishes. When the 7-day egg passage seed of HEP Flury virus was titrated for plaques in chick embryo fibro-

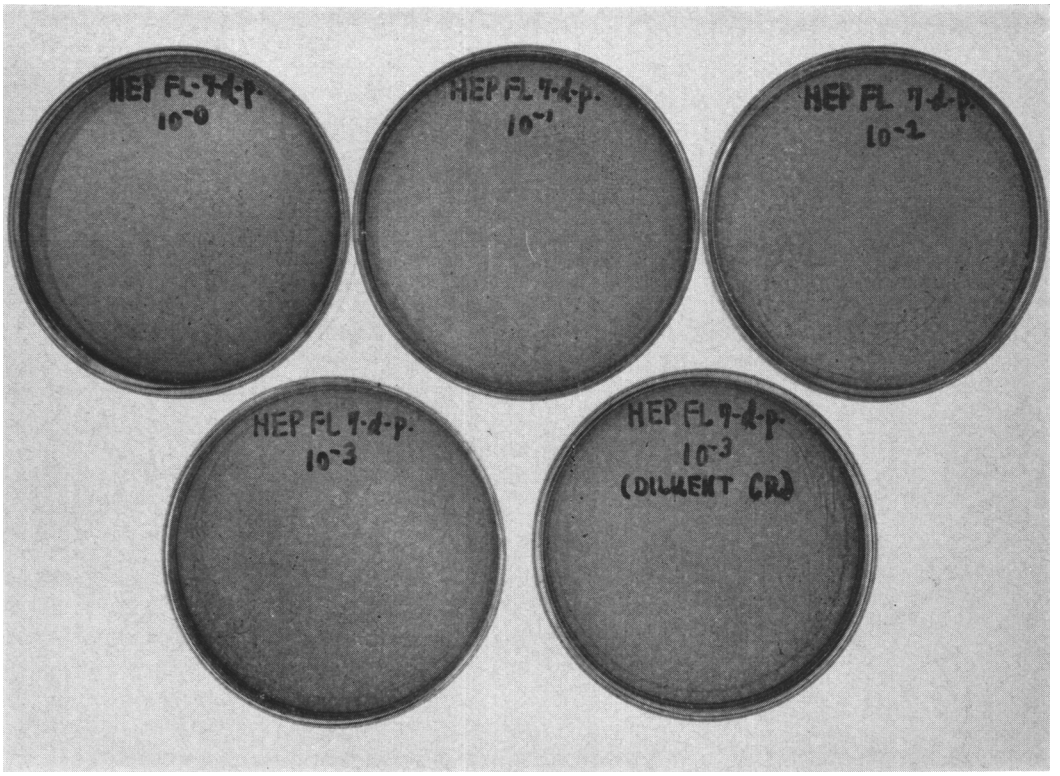

FIG. 1. Autointerference of HEP Flury strain of rabies virus appearing in plaque dishes after infection at a high multiplicity. A seed virus was diluted with yolk-saline and with a 20% emulsion of normal chick embryo brains in parallel, and dishes of chick embryo fibroblast monolayers were given the undiluted to 10^{-3} dilutions made with yolk-saline and the 10^{-3} dilution made with the normal chick embryo brain suspension (diluent control).

blasts, dishes receiving the undiluted seed developed no visible plaques, while those receiving higher dilutions showed confluent to scattered plaques depending upon the amount of virus inoculated. Microscopic examination revealed that almost all cells in the dishes given the undiluted seed virus stained red with neutral red. An example of such a titration is shown in Fig 1. In this test, a parallel set of dishes was inoculated with the same seed virus diluted in a 20% emulsion of normal 12-day chick embryo brains, but the development of plaques was similar in the two groups, proving that the above inhibition of plaques was not due to the presence of tissue components in the inoculum. Such a phenomenon was never encountered with other plaque-forming viruses such as vaccinia or WEE. The autointerference of rabies virus was observed with the chick embryo cell passage line of HEP Flury virus, too. Repeated test demonstrated that infection at a moi higher than 0.1 invariably resulted in such an autointerference.

Interference with vaccinia and WEE viruses occurring in monolayer dishes pretreated with a heavy concentration of HEP Flury virus. A group of monolayer dishes was infected with 0.2 ml per dish of HEP Flury virus at a moi of 0.3, and after one hour's incubation at 37°C washed once with PBS. Maintenance medium was then added to the dishes, which were subsequently incubated at 35°C for 24 hours. Control dishes were inoculated with an emulsion of normal embryonic brains in place of virus and treated likewise. These dishes were used for plaque titration of vaccinia, WEE, Sindbis and the homologous HEP Flury viruses. As indicated in Table I, interference was observed with vaccinia and WEE viruses, though to a lesser extent than with the homologous virus, while

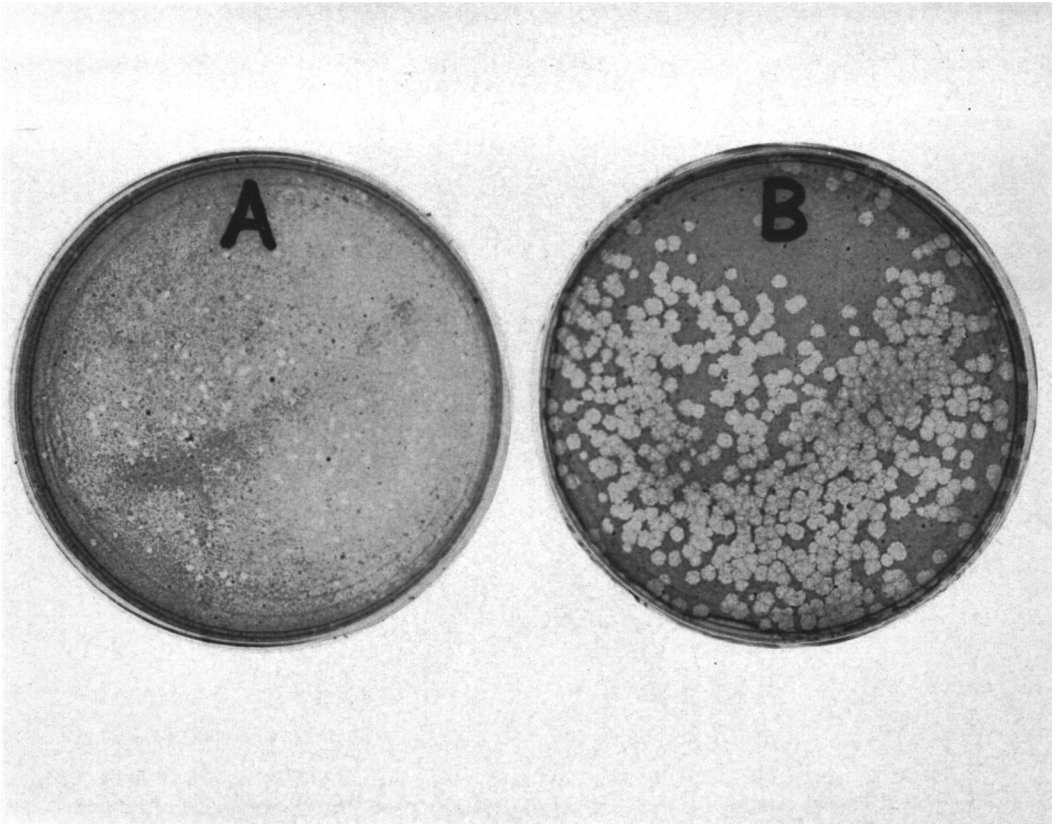

FIG. 2. Plaques of vaccinia virus appearing in chick embryo cells pretreated with a heavy concentration of HEP Flury virus (A) and in control cells pretreated with a normal chick embryo brain suspension (B).

Sindbis virus was not interfered with. In the case of vaccinia virus, not only the plaque number but also the plaque size was markedly reduced (Fig. 2).

It was suspected that interferon might be present in the original seed virus suspension. Therefore, the following experiment was performed. A seed suspension of HEP Flury virus was divided into 4 portions. One was subjected to quick dialysis to obtain interferon, the second was heated at 56°C for 30 minutes, the third was added with one-tenth volume of an immune rabbit serum and incubated at 37°C for one hour, and the last portion was left untreated as control. Then a test similar to the preceding one was done using these 4 portions and, as another control, maintenance medium as pretreating material and vaccinia virus as the indicator. As shown in Table II, none of the inactivated

TABLE I. Numbers of Plaques of Different Viruses Appearing in Monolayer Dishes of Chick Embryo Fibroblasts Pretreated with a Heavy Concentration of HEP Flury Strain of Rabies Virus.

Virus	Pretreated with	Dilution of virus 10^{-3}	10^{-4}	10^{-5}
Vaccinia	Virus*	136	12	2
	Control†	TNTC‡	45	7
WEE	Virus	90	10	1
	Control	TNTC	26	5
Sindbis	Virus	Conf§	TNTC	14
	Control	Conf	TNTC	19
Homologous HEP flury	Virus	0	0	–
	Control	52	6	–

* HEP Flury virus given at a moi of 0.3.
† A 20% emlusion of normal chick embryo brains.
‡ Too numerous to count.
§ Confluent plaques.
Numerals are plaque numbers averaged from 3 dishes.

TABLE II. Effects of Various Treatments of HEP Flury Virus Upon Its Capacity to Induce Interference with Vaccinia Virus in Chick Embryo Cells.

Pretreatment of cells with	moi	Dilution of vaccinia virus 10^{-2}	10^{-3}	10^{-4}
Active virus	.18	95	5	1
Heated "	0	TNTC*	35	8
Acid-dialyzed virus	0	TNTC	25	3
Neutralized "	0	TNTC	32	2
Maintenance medium	0	TNTC	39	4

* Too numerous to count.
Numerals are plaque numbers averaged from 3 dishes.

seeds induced any appreciable interference, while a marked intereference was seen in the cultures infected with active virus.

Autointerference of HEP Flury virus occurring in bottle cultures. An undiluted seed of HEP Flury virus and its 10^{-3} dilution were inoculated into 2 groups of bottle cultures of chick embryo cells, and at daily intervals 2 bottles randomly selected from each group were used for titration of fluid phase virus. The fluid pools were then subjected to acid dialysis to extract interferon. The resulting fluids were stored in a deep freezer and tested at one time for interferon activity with vaccinia virus. The result as depicted in Fig. 3, demonstrates that virus yields were similar in the two groups, but production of interferon was earlier and higher in the group infected with the undiluted seed (moi = 0.5) than in that infected with 10^{-3} diluted seed (moi = 0.0005). The interferon titers during the first 2 days were generally low, but when the homologous HEP Flury virus was used as an indicator, similar titers were obtained. Perhaps tne assay method was not very sensitive, or interferon was diluted by the fluid in the bottle culture. In the next experiment, 5 groups of bottle cultures were inoculated with different dilutions of a seed virus, and virus yields in the fluid were examined after 2 days' culture. The result recorded in Table III indicated that a maximum yield was obtained when the moi was 0.015, and higher concentrations of the seed virus resulted in reduced amounts of virus produced.

TABLE III. Virus Yields in Bottle Cultures of Chick Embryo Cells 2 Days After Infection at Different Multiplicities (moi).

moi	Virus in fluid* PFU/ml
1.5	2.2×10^6
.15	8.6×10^6
.015	2.6×10^7
.0015	3.2×10^6
.00015	4.0×10^5

* Pool of fluids from 2 parallel bottles titrated 48 hr following infection.

Discussion. Data have been available which indicate occurrence of suppression of virus replication in cells heavily infected with WEE virus(8), parainfluenza virus(9), NDV (10) and SV 40(11). However, in no case was the autointerference so marked as was observed in the present case of HEP Flury strain of rabies virus, which is characterized by a complete absence of viral plaques in monolayer cells leaving a marked interfering capacity against other viruses as well as against the homologous virus. In this connection, it is of interest that HEP Flury virus causes a marked autointerference in the brain of hamsters and guinea pigs(2).

Although these data leave no doubt that the autointerference of rabies virus in chick embryo fibroblasts is due to production of in-

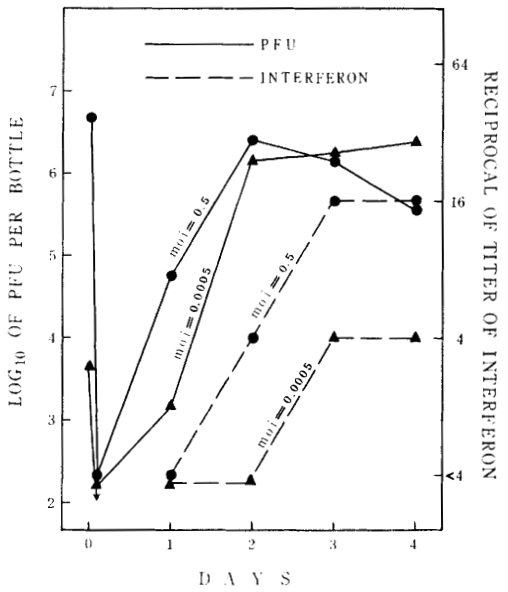

FIG. 3. Viral growth and production of interferon in bottle cultures of chick embryo fibroblasts after inoculation of an undiluted (moi = 0.5) and 10^{-3} diluted (moi = 0.0005) seed of HEP Flury virus.

terferon caused by a massive infection of cells by the virus, the mechanism underlying this phenomenon is not easy to understand. If such an autointerference should result from a simultaneous infection of adjacent cells, then plaque formation by this virus would have never occurred, because each infected cell releases newly produced virus at the end of the growth cycle which infects many neighboring cells almost at the same time. An explanation of this phenomenon, therefore, may be that one PFU of this virus corresponds to many virus particles, and consequently in an infection of cells at a moi of higher than 0.1 all cells are attacked by many non-infectious virus particles resulting in a von Magnus phenomenon(12). Alternatively, an interferon inducer as detected in the NDV-L(MCN) cell system(10) may be present in the original seed virus suspension or be produced on infection of the cells. What mechanism really operates in the autointerference requires further investigation.

Summary. When HEP Flury strain of rabies virus was inoculated onto chick embryo fibroblasts at a multiplicity of higher than 0.1, no plaques were formed and almost all cells remained stainable with neutral red. Monolayers pretreated with such a concentration of HEP Flury virus showed interference with plaque formation by vaccinia and WEE viruses. This was not due to presence of interferon in the seed virus suspension, nor to the presence of tissue components in the inoculum. In bottle cultures of chick embryo cells, virus growth after infection at higher multiplicities was accompanied by production of a considerable amount of interferon, and the highest virus yield was obtained when the multiplicity of infection was about 0.01.

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Liver Metabolism and Toxicity of Thiophosphate Insecticides in Mammalian, Avian and Piscine Species.* (31497)

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Organic phosphorothionate or phosphorodithioate insecticides are toxic to animals by virtue of their capacity to inhibit acetylcholinesterase. The actual inhibitors of cholinesterase are the oxygen analogues which are metabolically formed from the parent insecticides *in vivo*(1,2). The relative susceptibility of various species to poisoning by these insecticides might, therefore, be determined in whole or in part by the rates at which the oxygen analogues accumulate in the different organisms. This accumulation will be dependent not only upon the rates of formation of the oxygen analogues but also upon the rates at which the parent insecticides or their oxygen analogues are destroyed by tissue

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