HOMOLOGOUS INTERFERENCE BY ULTRAVIOLET INACTIVATED VIRUS IN NEWCASTLE DISEASE VIRUS

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Marcel Albert Baluda

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ABSTRACT

The present study concerns a quantitative analysis of the interference between the irradiated and the active Newcastle disease virus.

The inactivated particles adsorb to the surface of the cells and do not proceed any further. This union induces surface changes which make it impossible for a superinfecting active particle to penetrate into the host cell and to initiate the production of new virus. The more UVI particles that are adsorbed, the faster the interfering reaction occurs.

In 50 per cent of the cells, however, interference is not complete; these cells can be superinfected provided the multiplicity of the superinfecting virus is high. The effect is equivalent to having on the average four per cent of the total surface of the cell unaffected by the changes induced by the inactivated virus.

The interfering reaction is dependent at all times upon the presence of the unmodified UVI particles at the critical sites - exposure of the interfered cells to specific anti-NDV serum eliminates interference. However, in 50 per cent of the cells interference becomes irreversible 30 minutes after the attachment of UVI virus. Whether this irreversible interference involves more profound cellular changes or depends upon the physiological state of the cells at the time of infection is at present unknown.

Eventually, the union between UVI virus and cellular site is broken with the subsequent return of the cell to susceptibility to

infection. This loss of resistance occurs spontaneously from 26 to 60 hours after exclusion has been induced.

The superinfecting virus which does not initiate infection is destroyed after its adsorption to the lung cell. A cell where interference has been removed after superinfection with active virus must be infected a second time in order to yield progeny virus.

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TABLE OF ABBREVIATIONS

CW: A nutrient solution made up of amino acids and vitamins

DNA: Deoxyribose nucleic acid

EE: Chicken embryo extract

ES: Earle's saline

HA: Hemagglutinating unit

ID_{50}: The infective dose giving an end point of 50 per cent mortality

LA: Lactalbumin hydrolysate

LAY: LA plus yeast extract

Lee: Lee strain of Influenza B virus

Mel: Melbourne strain of Influenza A virus

NDV: Newcastle disease virus

NIH: Non-infectious hemagglutinin

PBS: Phosphate-buffered saline

Pfu: Plaque forming unit

PR8: PR8 strain of Influenza A virus

RBC: Red blood cell

RDE: Receptor destroying enzyme

RNA: Ribose nucleic acid

$T_{1, 2, \ldots 7}$: Any one of the T series of bacteriophages attacking *Escherichia coli* B

UV: Ultraviolet light

UVI: Ultraviolet irradiated

INTRODUCTION

A. General remarks:

The development of new and more accurate methods of investigation of virus-host interaction at the cellular level (1) has made possible new attempts at solving the problem of interference. Interference consists in the inhibition of the growth of one virus in cells infected by another virus. When interference takes place, the process of virus synthesis in the infected cell is disturbed at some stage. Therefore, a knowledge of this mechanism may afford a tool for studying the reproductive processes involved in the synthesis of new virus, especially at the early steps of infection.

An understanding of the phenomenon of interference is also required for adequately carrying out experiments of genetic recombination. Indeed, recombination of genetic markers among different virus particles depends on the non-occurrence of mutual exclusion.

The simplest way to study interference appears to be through the use of inactivated virus as the interfering agent. Since such an interfering agent cannot multiply, there is no need for differentiating the progeny of the interfering virus from that of the superinfecting virus in the yield. The use of the same type of virus as the interfering and superinfecting agent (homologous interference) seems also preferable because they must follow at least at the beginning an identical path in the infected cell. Preliminary experiments in this investigation have confirmed the view generally held by students of interference (2) that ultraviolet irradiated (UVI) virus is the

best available interfering agent, the results being invariably reproducible.

For a quantitative study, the use of homogeneous cell suspensions as the host for virus growth is imperative. Only in such a system can the number of susceptible cells and the number of virus particles adsorbed per cell be determined. The kinetics of infection and production of new virus can therefore be rigorously defined.

In addition, a virus must be chosen that can be assayed by plaque count (1).

A system which satisfies those requirements consists of the virus of Newcastle disease and chicken embryonic lung cells grown in tissue culture.

B. The NDV-lung cell system:

Newcastle disease virus (NDV) and chicken embryonic lung cells will be the virus-host system used throughout this investigation. This system possesses certain properties which make it an ideal tool for this particular study. The essential properties of the two components of this virus-cell system will now be reported.

Newcastle disease is an epizootic infection of fowl characterized by viremia, focal necroses in the viscera and hemorrhages, especially in the respiratory and alimentary tracts. Death usually ensues on the sixth to eighth day, but the mortality is comparatively low in adult birds. The malady occasionally attacks human beings (3) manifesting itself as a superficial conjunctivitis and probably results from viral infection by contaminated fingers.

The etiologic agent belongs to the Myxovirus group (4) comprising the influenza and related viruses i.e. fowl plague, Newcastle disease and mumps. The characters defining the group are a spherical form with a diameter of approximately 80 mμ (5)* and the capacity to act as hemagglutinating agents brought about by the ability of the virus particles to adsorb to certain mucoproteins (6).

Work on the chemical composition of the infective particles among the Myxoviruses has been mostly concerned with the influenza viruses. The virus particles have a high lipid content in the range 30 to 40 per cent of dry weight (5). Ada and Perry (15) in 1954 found no significant amount of DNA and only approximately .8 per cent of RNA; the remainder is protein. Miller (16) in 1956, however, has reported the presence of about .1 per cent of DNA which cannot be denied recognition. Very recently, Franklin et al (17) (1956) have shown that the nucleic acid composition of NDV is similar to that of influenza virus. These authors have also shown that the phospholipid fraction of NDV is essential for the infectivity of the virus particles.

NDV, similarly to the other members of the Myxovirus group, displays the property of agglutinating certain red blood cells (hemagglutination). One hemagglutinating unit (HA) is the highest dilution of virus capable of agglutinating all the cells in one ml. of an erythrocyte suspension containing .125 per cent of cells - approximately 8×10^6 cells. One hemagglutinating unit corresponds to about 3×10^6 plaque forming units (pfu).

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* Larger sizes reported in the literature appear to be caused by deformation during drying (7,8,9,10,11,12,13,14).

Hemagglutination results from mutual adsorption of viral and cellular surfaces with the virus forming bridges to hold the cells together (13). The adsorptive sites on the virus surface are assumed to be of protein composition (18). The cell receptors are of mucopolysaccharide nature and the enzymatic action of the virus is on the polysaccharide unit of the complex (19). The distribution of the muco-protein receptors on the red cell surface is not known, but they are probably uniformly distributed over the surface. In fact, it is possible to have the red cell surface covered by virus particles packed close together as seen in the electron microscope; with low concentrations of the virus particles, their distribution over the cell surface is random (13).

After adsorption of the virus to the erythrocyte surface, there is a characteristic elution of the virus into the medium (20). In the case of NDV, adsorption to red blood cells occurs equally well at all pH levels between 6 and 8, but elution is minimal at pH 6 (17). Elution of NDV takes place very rapidly above 30°C and is complete within 30 minutes at 37°C. Moreover, this virus is capable of lysing fowl erythrocytes (21) and is reported by Burnet (22) to have the most powerful enzymatic action among the Myxo-viruses. After elution, the virus itself can adsorb to fresh cells indefinitely but the red cells are so modified that they can no longer adsorb the same virus (20). Such cells also show a decrease in their electrophoretic mobility characteristic for the virus used (23,24). Cells treated with NDV can, however, be agglutinated by influenza viruses (25). This led to the

discovery of a "receptor gradient" where the viruses can be arranged in a linear order (24) - any virus in the series renders red cells inagglutinable by all viruses preceding it in the series, but leaves the cells agglutinable by all viruses following it. The order is as follows: mumps, NDV, Influenza A, Influenza B and Swine influenza.

Burnet et al (26) in 1946 extracted from Vibrio cholerae cultures a soluble enzyme which they called "receptor destroying enzyme" (RDE) because of its ability for removing virus receptors from red cells. This enzyme was shown to be isodynamic with the myxoviral enzyme (25,27). RDE renders erythrocytes inagglutinable by any of the Myxo-viruses, thus placing itself at the top of the receptor gradient (26). In addition, RDE, NDV and Swine influenza virus are thought by Stone and Ada (24) to be able to act on a second set of cell receptors which are not concerned with the adsorption of viruses, although susceptible to destruction by viruses. Viruses of this group rendered non-infective by heat treatment (28) or ultraviolet irradiation (29) can still hemagglutinate red cells. The hemagglutinin titer can, therefore, be used as a measure of the number of non-infectious particles present in a given viral suspension.

Heated influenza virus which has lost its enzymatic activity can adsorb to but cannot elute from erythrocytes. Treatment of such a virus-red cell complex with RDE or homologous antiviral serum is found to free the adsorbed inactivated virus particles (6).

The following conclusions may be drawn about the adsorption of NDV to red blood cells. When the virus collides with a red cell,

electrostatic forces mediated by ions, especially Ca^{++}, bring several pairs of groupings into juxtaposition (30,31). This provides a holding force to maintain virus and cell together and also an opportunity for oriented union between enzyme and substrate, resulting eventually in the destruction of the receptors and elution of the virus particle (32).

It is tempting to transfer the findings obtained with red cells to susceptible host cells capable of producing progeny virus, with the obvious exclusion of the last step, namely, the elution of the virus. Under normal conditions of infection, the virus disappears soon after its attachment to the cell and becomes undetectable by any method used so far (33).

As described later under Methods, the lung cells used in this investigation were grown in tissue culture. They form layers on the glass surface essentially one cell thick with occasional clumps. The cells are spread on the glass and are very thin. When the layers are disrupted with versene, the single cells assume a spherical shape with a diameter of approximately 12 microns. The virus receptors of the lung cells can be destroyed with RDE but are regenerated within 2 or 3 hours after the removal of this enzyme (34). NDV cannot adsorb to lung cells, the receptors of which have been destroyed with RDE.

The NDV-lung cell system has the invaluable advantage over all other previously used systems that the fraction of cells actually infected and liberating new virus can be determined. The assay of such cells can be carried out by plaque count since the plating of infected cells before they start releasing virus gives rise to plaques in a manner identical to that of free active virus particles.

C. Interference phenomena in the literature:

Interference phenomena were first described between plant viruses by Wingard (35) in 1928. Plants of Nicotiana tabacum recovering from infection with tobacco ring spot virus and still carrying the virus did not come down with the disease when reinfected with the same virus. These plants were likewise not susceptible to closely related viruses such as tobacco mosaic and cucumber mosaic viruses.

Extensive work on interference was carried out with bacterial viruses. Since these viruses have become a model and reference system in basic virology, the information available from bacteriophage work will now be presented first.

1. Bacterial viruses:

Burnet and Lush (36) in 1936 found that after exposure to the weakly lytic phage C a staphylococcus culture became resistant within an hour of contact, to C as well as to an intensely lytic derivative phage C'. These authors concluded that the bacterial surface had changed so that it no longer adsorbed either phage. A similar observation was reported by White (37) in 1937 with two lysogenic phages of Vibrio cholerae. White presented the hypothesis that the less virulent phage establishes itself on the phage receptors of the bacterium and blocks the entry of the superinfecting phage.

Mutual exclusion was found to occur when bacteria were simultaneously or consecutively infected by two dissimilar virulent phages (Delbruck and Luria, 1942) (38). A bacterium infected with active T_1 or T_2 will liberate only T_2 after a latent period equal to the

normal period of virus T_2. No growth of T_1 takes place in these bacteria. Suppression of T_1 growth is complete if virus T_2 is added up to 2 minutes after T_1; with larger time intervals, suppression disappears rapidly. This effect is probably caused by the different rates of adsorption and infection of these two viruses--in 5 minutes 75 to 80 per cent of free T_2 is adsorbed, whereas in the same time only 45 to 58 per cent of T_1 is adsorbed. In no case, however, are both phages liberated from the same bacterium. Moreover, a single particle of T_2 was found sufficient to suppress completely the growth of T_1. The authors interpreted the phenomenon in terms of a key enzyme present in a single copy in each cell necessary for the production of phage and completely monopolized by the first virus.

Luria and Delbruck (1942) (39) have further shown that T_2 irradiated with ultraviolet light retains its ability to interfere with the growth of T_1 and that a single UVI phage is sufficient to do so. Furthermore, it was demonstrated that UVI T_2 can interfere with active T_2 if given prior to the active homologous virus. However, T_2 could grow in some bacteria infected with UVI T_2. Luria and Delbruck also found that UVI T_1 does not interfere with active T_2 nor active T_1.

The "key enzyme" hypothesis was dropped by Delbruck (40) in 1945 when he found that mutual exclusion operates with an efficiency better than 99 per cent with T_1 and T_7. (T_7 occupies an intermediate position between T_1 and T_2 with respect to interference). He argued that a key enzyme would have to duplicate some time before each cell division and that it was not reasonable to assume the presence of only

one key enzyme in more than 99 per cent of the cells in a growing culture. Delbruck then advanced the "penetration hypothesis"; he assumed that the first phage which penetrates into the cell causes the entire cell membrane to become impermeable to other phages. He found, however, that the virus which is excluded from growth takes a hand in the course of events; the excluded virus depresses the yield of the successful virus (Depressor Effect), if the two phages are dissimilar. The depressor effect diminishes gradually when the time interval between the addition of the two viruses is increased. Beyond a critical time interval there is no depressor action. Antivirus serum added $3\frac{1}{2}$ minutes after adsorption of a virus diminishes its depressor action. This effect is interpreted by the author as competition for a common substrate between the virus which penetrated the cell and the excluded virus on its surface. However, this phenomenon could be explained now by "lysis from without" which was unknown at the time.

The "penetration hypothesis" received strong support from the work of Lesley, French, Graham and van Rooyen (41) (1951) for the large phages T_2, T_4, T_6 and the unrelated phage T_5 . When Escherichia coli B is infected with any of these phages and a few minutes later infected with T_2 labeled with P^{32}, the labeled T_2 is rapidly degraded into low molecular weight fragments. The first infection stimulates the bacterium to break down the superinfecting phage. The small phages T_1, T_3 and T_7 do not stimulate the bacterium to cause breakdown.

Exclusion of an homologous bacteriophage does not occur if the interfering and the superinfecting virus attach to the cell almost

simultaneously. Hershey (42) in 1946 discovered that the related phages T_2 and T_{2r} can multiply together in the same bacterium and used this finding to study the recombination of genetic markers in phages. Dulbecco (43) (1952) studied exclusion between homologous viruses through the use of genetically marked bacteriophages. He found that bacteria infected with T_2 are stimulated to exclude the superinfecting phage T_{2r} arriving later and vice versa. The superinfecting phage is excluded in 50 per cent of the bacteria when the interval between infections is one minute. T_2 inactivated by ultraviolet light was also found to exclude superinfecting phage at the same rate as does active T_2. Moreover, Dulbecco showed that the stimulation to exclusion is dependent on processes requiring an external energy supply since there is no exclusion in resting bacteria in buffer. The latter author interpreted the stimulation to breakdown, the stimulation to exclusion and the destruction of the chromatinic bodies of infected bacteria (Luria and Human (44), (1950) as three aspects of the same UV resistant function of the virus involving "the rapid mobilization of a powerful enzyme system attacking nucleic acids".

New means of approach to the problem of exclusion were offered by lysogenic bacteria. Weigle and Delbruck (45) (1951) tested whether mutual exclusion occurs between a carried phage induced to mature and a virulent phage introduced from the outside at various times during the maturation period of the former. They studied the system of Escherichia coli K12 carrying λ and the lytic phage T_5. They found that λ in the prophage condition does not exclude T_5; however, there is no mixed yield

of λ and T_5 - or less than one per cent - in induced bacteria superinfected with T_5. A bacterium thus treated is capable of three alternatives: to yield T_5, to be "lysed from without" thereby liberating any mature λ particles present or to yield λ after completion of the maturation period. In this case λ is not excluded by a barrier to penetration since λ was already inside the bacterium and, in some cases, near completion of maturation. Weigle and Delbruck concluded that in this system exclusion occurs at a terminal stage of phage synthesis.

Interesting findings pertaining to exclusion occurred in the investigation of the functions exercised by the protein envelope, other than that of serving as a carrier for phage DNA. Herriot (46) in 1951 found that the T_2 membranes obtained by osmotic shock prevent multiplication of host bacteria and can cause lysis of the cells when present in sufficient numbers. Moreover, the ghosts have a range specificity similar to that of the virus from which they were derived. Bonifas and Kellenberger (47) in 1955 have shown that T_2 membranes contain a proportion of killer particles in which one particle can kill one bacterium. All the membranes, however, are capable of producing a reversible physiological effect which induces changes in the bacterial nucleus. This action is totally different from that brought about by intact T_2 and, in fact, bacteria treated with T_2 ghosts exclude active T_2 superinfecting them after a short time.

French and Siminovitch (48) (1955) have recently investigated this phenomenon in more detail. These authors found that 75 per cent

of T_2 membranes can be adsorbed to host cells as measured with S^{35}; of these, 10 to 35 per cent are killers. The remaining fraction of the ghosts which is adsorbed but does not kill the host arrests the division of the bacterium. These "ghost infected" bacteria do not support the development of a secondary infecting T_2 or T_1 particle, synthesize little protein and respire like phage infected bacteria. This situation prevails for about 60 to 120 minutes and then the bacterium recommences to multiply and behaves like a normal cell.

2. Animal viruses:

The extensive literature concerning interference in animal viruses has been adequately reviewed by Henle (2) in 1950 and the salient points brought out by Dulbecco (49) (1955) in his review. According to Dulbecco, exact quantitative data on interference are not available; nevertheless, some information can be extracted from a number of experimental data obtained using chicken embryonated eggs as the experimental host. In line with the present investigation, the following comments will be concerned only with interference between inactivated and active virus systems.

Influenza virus can be treated with heat (56°C, 37°C and 4°C), formaldehyde or ultraviolet light in such a way that it loses its infectivity but retains its interfering ability. The general view is that virus inactivated at 56°C is a poor interfering agent. Fazekas et al (50) in 1952 have shown that Lee Influenza B virus heated at 56°C for one hour under conditions which were not defined in detail causes only partial and delayed interference upon the growth of Mel Influenza

virus; the growth of the latter was only affected after 14 hours with only a ten-fold drop in titer. Fazekas and Edney (51) (1952) have statistically demonstrated from a study of the yields of progeny virus that one particle of Lee virus heated at 56°C for one hour is sufficient to interfere completely with the growth of active Mel virus added 24 hours later. On the other hand, Ziegler et al (52) in 1944 had found that heating infective allantoic fluid at 56°C destroyed the infectivity of PR8 and Lee viruses as well as their capacity to produce interference in approximately equal time. Henle and Henle (53) in 1944 also reported that heating infective allantoic fluid to 56°C or formalinization of PR8 and Lee viruses destroy most of the interfering capacity together with the infectivity; only residual interfering effects are demonstrated with such interfering agents.

Isaacs and Edney (54) (1950) have reported that Mel, PR8 or Lee virus heated at 56°C for one hour under conditions which completely destroy the viral enzymatic activity on ovomucin is a more efficient interfering agent than formolized Mel, PR8 or Lee virus. This heat inactivation was performed by adding two parts of 2 per cent citrate-saline and one part of borate buffer to six parts of infective allantoic fluid and heating the mixture at 56°C for one hour. Heated Lee was found to be a better interfering agent than heated Mel, which in turn was better than PR8 virus; the differences are, however, barely significant. The authors concluded that in this case interference is not caused by enzymatic destruction of the receptor substance of susceptible cells, in contrast to the hypothesis advanced by Ziegler et al

(52). In a second paper, the same authors (55) have found that the allantoic sac is capable of adsorbing a greater quantity of heated virus than the minimal interfering dose which they defined as the least amount of heated virus capable of preventing the appearance of hemagglutinin in eggs infected with 100 ID_{50} of challenge virus. They ruled out blocking of receptors as the interfering effect of the inactivated virus, although the interfering action of one minimal interfering dose could be overcome by increasing the size of the superinfecting inoculum to and above 10^8 ID_{50}. By antiserum titration and RDE treatment of infected allantoic sacs, these authors tried to show that entry of live virus into the allantoic cells is not prevented by their prior infection with heated virus. From unconclusive evidence they stated that interference is an intracellular phenomenon.

Virus inactivated by heat at 37°C and 22°C and by standing at 4°C was found to behave in a manner identical with that of UVI virus (52,54,56). Ziegler et al (52) (1944) have shown that PR8, Lee and Swine influenza viruses irradiated with ultraviolet light and used in amounts sufficient to infect all the cells of the allantoic membrane interfere completely with the growth of active heterologous virus. They also noted that interference took place even when the UVI and the active particles were injected simultaneously. These authors advanced the theory that interference might result from quantitative saturation of the receptor substance of susceptible cells.

Henle and Henle (57) (1944) have found that exclusion may be induced within less than ten minutes by UVI-PR8 preceding active Lee virus. The interfering activity of the UVI virus was shown to be

destroyed with increasing doses of UV light and also by mixing immune serum with the interfering agent prior to its injection into the egg (52,58). Henle et al (59) in 1947 reported that exclusion is induced within one minute or less between UVI-PR8 virus and active Lee virus by adding potent anti-PR8 rabbit serum one minute after the UVI virus and noting that it failed to inhibit the interfering effect. It is not clear, however, whether this lack of action by the serum upon the interfering ability arose because interference had already taken place or because the antiserum was merely unable to neutralize the UVI particles under the experimental conditions.

Henle et al (60) have reported that UVI-PR8 or UVI-Lee virus might have an effect upon the growth of the homologous active particles even when the interfering agent is added after the active virus. These authors claimed that there was a depression in the yield of progeny virus brought about by homologous UVI virus added one hour later, but not by heterologous virus. The degree of depression of the yield was found to decrease gradually with an increase in the interval between the two infections and also with a dilution of the interfering agent. However, if the cells were infected with a high multiplicity of the active virus, the inhibitory effect of the UVI virus was decreased or suppressed (Henle, 1949) (61). This strongly suggests that in the experiments with a low multiplicity of the interfering virus not all the susceptible cells had been infected.

Henle et al (59) have also noted that no measurable concentrations of hemagglutinin were produced when eggs were infected with

UVI-PR8 virus, injected with anti-PR8 serum to neutralize the unadsorbed particles one hour later and superinfected with active Lee virus two to six days later. In other words, interference was found to last up to six days in the cells of the allantoic sac. Henle and Henle (29) (1947) have found that UVI-PR8 virus may retard the development of the allantoic sac of seven to eight day old chicken embryos. On prolonged irradiation of the virus, this effect was destroyed although the UVI particles retained their ability to hemagglutinate erythrocytes and to adsorb onto the cells of the allantoic sac. They, therefore, concluded that a further step beyond adsorption is required to induce interference. However, these authors did not show that inhibition of cell growth is closely associated with the interfering ability of the UVI particles. Henle and Henle have also reported that additional particles of influenza virus can adsorb to host cells which have been rendered resistant to infection by UVI virus.

In conclusion, it may be said that experiments carried out with bacteriophages have by far yielded the most reliable results. From this work, it is apparent that one particle of UVI phage is sufficient to interfere with the multiplication of a superinfecting homologous or heterologous active virus attaching to the same cell a short time later. Exclusion of the superinfecting phage takes place very rapidly e. g. in one minute in 50 per cent of the bacteria infected either with active or UVI-T_2. Dual infection occurs only with closely related phages and only if they infect the bacterium almost simultaneously. The exclusion brought about by the attachment of one empty phage membrane, i.e. devoid of DNA, suggests that the interfering phenomenon

takes place at the bacterial surface as was suggested by Delbruck (40). Moreover, it appears that the penetration of the interfering agent into the bacterium is not a prerequisite for the induction of exclusion. The breakdown of the superinfecting phage at the bacterial surface in the experiments reported by Lesley et al (41) also favor this hypothesis. Exclusion induced by an homologous UVI interfering agent seems to be a surface phenomenon. However, the exclusion of the lysogenic phage λ by the virulent phage T_5 infecting the bacterium after λ has been induced to mature points to the existence of a second type of interference which takes place at a later stage in the infectious process. There are then two different kinds of interference; one type occurs at the bacterial surface while the second one takes place intracellularly.

The complicated experimental conditions and the incomplete knowledge of all the necessary variables of the system have rendered difficult, if not impossible, a correct interpretation of most experiments performed with animal viruses. Few experiments have been performed under conditions which permit a quantitative interpretation; when they could be properly interpreted, they have yielded results about interference very similar to those obtained in the work with bacterial viruses.

It has been suggested that one particle of heated influenza virus may be sufficient to inhibit the production of progeny of a superinfecting, active, heterologous virus adsorbing later. Whereas heated influenza virus induces interference slowly and incompletely, UVI influenza virus appears to induce exclusion very rapidly and in all

the cells to which the virus has adsorbed.

Some evidence has been presented which indicates that interference is not caused by the destruction of cellular receptors nor to their saturation. However, since the problem of the penetration of the interfered virus remains unsolved, no conclusion should be derived about the intracellular nature of this phenomenon.

MATERIALS AND METHODS

Solutions and Media

Phosphate-buffered saline (PBS), trypsin solution (.25 per cent trypsin in PBS), neutral red solution (1:10,000), Earle's saline (ES), chicken embryo extract (EE) (1:1 in ES) and ox serum were prepared as described by Dulbecco and Vogt (62).

Versene in PBS: Di-sodium versenate (di-sodium salt of ethylene diamine tetra acetic acid-di-hydrate) - Bersworth Chemical Co., Framingham, Mass. - .2 gm., NaCl 8.0 gm., KCl .2 gm., Na_2HPO_4 1.15 gm., KH_2PO_4 .2 gm., double distilled water to make up 1000 ml. Autoclave 20 minutes, cool, then add .1 gm. of streptomycin and 100,000 units of penicillin.

Lactalbumin hydrolysate (LA): To regular ES was added per 1000 ml.: lactalbumin hydrolysate (enzymatic) - Nutritional Biochemical Corp., Cleveland, Ohio - 5.0 gm., antimycotic (n-butyl p-hydroxybenzoate)- The Matheson Co., Inc., Joliet, Ill. - .2 gm. Bubble CO_2 to lower the pH to 6.6, then filter.

LA-Yeast (LAY): LA to which was added 1.0 gm. of yeast extract (dehydrated) - Difco.

Tissue culture fluids: The cell layers were grown in either LAY plus four per cent ox serum or in CW (a nutrient solution made up of amino acids and vitamins described by Charity Waymouth (63)) plus four per cent ox serum. The following media were used in the course of

an experiment: either NMI (10 per cent ox serum and five per cent EE in LAY) or NMII (LAY alone).

Agar overlay: The nutrient agar overlay was prepared as previously described (62) except for the following modifications. A volume of 6 ml. of overlay was used containing .85 per cent of unwashed Difco agar in LAY to which was added .6 ml. of EE.

Virus

Two strains of NDV were used. Some of the earliest work was carried out with the Beaudette strain (B-NDV) (64) supplied by Dr. F. B. Bang of Johns Hopkins University. This strain was soon replaced by the strain L-Kan 1948 (65) supplied by Dr. C. A. Brandly of the University of Wisconsin. The latter strain will be designated from now on as K-NDV. It was preferred to the former virus because of its greater heat stability and its capacity to produce higher titers of progeny virus. K-NDV has the following half-lives in PBS at pH 7.4 at 56°C, 45°C and 37°C: 5 minutes, 156 minutes and 24 hours respectively. The original titer remains unchanged for at least one month at 4°C. One infected lung cell is capable of yielding 1000 new particles of this virus after an incubation period of 24 hours at 37°C in LA.

Preparation of viral stocks

Stocks were prepared by inoculating into the allantoic cavity of 10 to 11 day old chicken embryonated eggs 0.1 to 0.2 ml. of a 10^3 dilution of a previous viral stock. After a period of incubation

of 48 hours at 37°C, all the embryos were dead and, after several hours of chilling at 4°C, the allantoic fluids were harvested. The fluids were then spun in an International centrifuge size 1 at 3000 rpm for 5 to 10 minutes; after this, they were centrifuged at high speed in a Spinco model L ultracentrifuge (25,000 rpm with a rotor #30 for 30 minutes). The pellet was resuspended in the initial volume or less of PBS and broken up by vigorous pipetting with an automatic 2 ml. pipette. After standing overnight in the refrigerator, the viral suspension was spun again at low speed to remove any material which had not dissolved. The final supernate was then used as the immediate source of virus and stored at 4°C. Fresh stocks were prepared about every third week. The stock titers varied from 2 to 8 x 10^9 plaque forming units (pfu) with a pfu to HA ratio of about 2.7 x 10^6. This indicates that all or nearly all the virus particles are infectious as well as hemagglutinating since one HA unit is equal to approximately 3 x 10^6 pfu.

Preparation of tissue cultures

Two types of cell cultures were used throughout this investigation - chicken embryo cells and chicken embryo lung cells. The former were used for routine plaque assay of both free virus and infected cells while the latter cells being fairly homogeneous and the natural host for NDV were used as the experimental host.

1. Chicken embryo cells:

The method for preparing these monolayer cultures has been described by Dulbecco (1).

2. Chicken embryo lung cells:

Sixteen day old chicken embryonic lungs were aseptically removed into warm PBS and rinsed in PBS to remove blood and debris. The lungs were then cut into about six pieces each. Following a second rinsing, the pieces were incubated for approximately ten minutes at 37°C in PBS containing .25 per cent trypsin. The tissue was then vigorously pipetted twenty to thirty times with a 2 ml. automatic pipette, until an homogeneous preparation consisting entirely of clumps of twenty to thirty cells each was obtained. These clumps were washed twice in PBS by centrifugation at 1000 rpm for two minutes in an International size 1 centrifuge and finally resuspended in LAY containing 10 per cent of ox serum and five per cent of embryo extract. A volume of 10 ml. of this cell suspension containing a total of 2×10^6 clumps were used to start a culture in a 100 mm. pyrex culture dish. The cultures were incubated at 37°C in a well humidified incubator. The pH in the unsealed Petri dishes was controlled by the continuous flow of a four per cent CO_2 in air mixture inside the incubator. These cultures could be used for virus growth after two days of incubation at which time the cells had spread to form a continuous layer of epithelium on the bottom of the plate. The layers could be used up to the sixth day after the start of the culture.

Plaque assay of the virus and infected cells

The plaque assay for animal viruses has been described in detail by Dulbecco and Vogt (1954) (62). The following modifications were used, however. A total of 6.6 ml. of nutrient agar overlay was

used containing LAY instead of ES. The fibroblast layers were used at 24 hours and the plaques could be counted after 3 days of incubation. The plaques averaging 2 to 3 mm. in diameter could be clearly seen without neutral red staining.

Infected lung cells when plated on assay monolayers produced plaques which were indistinguishable from those formed by free virus. They were therefore assayed in an identical manner.

Hemagglutination titration

HA titrations were carried out by the Salk pattern technique (Salk, 1944) (66) with some modifications designed to destroy HA inhibitors and viral enzymes suggested by Granoff (1955) (67). A volume of .1 ml. of a virus suspension was diluted into .7 ml. of PBS and .1 ml. of .1 N $NaIO_4$ was added. This mixture was adsorbed for 30 minutes at 4°C and the excess periodate was neutralized with .1 ml. of 40 per cent of glucose. Serial two-fold dilutions of the mixture were made in PBS and to .5 ml. of this suspension was added .5 ml. of .25 per cent washed chicken erythrocytes in PBS. Patterns were read after 3 to 4 hours of standing at 4°C. The readings of +, ± or — were read as in Salk's method; however, ± instead of + was taken as the end point. Where + proved to be the observed end point followed by —, ± was taken as the geometric mean of the two values.

Irradiation of the virus

The viral suspension in PBS was exposed in a volume of 2 to

2.5 ml. in an open 60 mm. Petri dish to UV irradiation by a Westinghouse germicidal lamp from a window 10 cm. long by 5 cm. wide. The intensity at the sample was of the order of 20 ergs per $mm.^2$ per sec. and about 80 per cent of the energy emitted was in the wave length of 2537 A°. The fluids were shaken every 40 seconds to insure proper mixing.

Antiserum

Rabbits were inoculated over a period of 2 months with 14 alternating intravenous and intramuscular injections of active NDV. Normal and anti-NDV sera were obtained by cardiac puncture before the injections and one week after the last injection respectively.

Antibodies to normal chicken embryonic tissues were removed by incubating the serum with about 10^9 suspended chicken embryonic cells for 7 hours at 37°C and for 24 hours at 4°C. The cells were then centrifuged and the serum kept frozen at -18°C.

This serum had a K value (68) of 30. At a dilution of 1:100 in PBS in 15 minutes at 37°C, it reduced the infectivity of a given amount of NDV to one per cent.

Receptor destroying enzyme (RDE)

A crude filtrate of a Vibrio cholerae culture was obtained through the courtesy of Dr. Harry Rubin. It had a titer of 800 units per ml. A unit of RDE was defined as the highest two-fold dilution which prevented the agglutination of .5 per cent RBC by eight HA units of NDV.

General experimental procedures

All the experiments performed may be summarily broken down into two general types. The first type will be called infection on the plate, the second type infection in suspension.

1) Infection on the plate:

In this case the layer of lung cells was infected while still adhering to the glass surface of the culture plate. The nutrient medium was sucked off and the cell layer washed twice with warm PBS. One ml. of the desired inoculum was then deposited on the layer and permitted to adsorb for a given time. The inoculum was then removed and the cells were again washed several times with warm PBS. After the last washing, the cells were subjected to whatever treatment the experiment called for - treatment with serum, RDE, incubation in nutrient medium, etc. If the experiment required superinfection of the cells with a second inoculum, the above procedures were repeated. After the final rinsing of the culture with PBS, a volume of 5 ml. of warm versene was then added to the layer and permitted to act for five minutes. The layer became disrupted and the cells dispersed in suspension. This cell suspension was then transferred to a 12 ml. conical centrifuge tube and pipetted vigorously 10 to 15 times until a preparation consisting entirely of single cells was obtained. The cells were then centrifuged at 1200 rpm in an International centrifuge, size 1, for 2 minutes and resuspended in 5 ml. of PBS lacking Ca^{++} and Mg^{++} ions to prevent clumping. This washing procedure was repeated twice and the cells were then resuspended in 2 to 4 ml. of Ca^{++} and Mg^{++} free PBS to give a final concentration of approximately 10^6 cells per ml. It was later found that all these

steps could be done in versene.

The total number of cells present in such a suspension was then determined by direct count in a Neubauer counting chamber. Both sides of the chamber were used and the cells in all the 25 quadrants were counted. The number of cells counted was always at the very least higher than sixty; therefore, this gives a maximal variation of about 13 per cent in the number of cells counted.

The cells were serially diluted in PBS to find the concentration which gave approximately 100 pfu and then assayed by plating.

The last cell suspension (before dilution) was centrifuged once more to sediment all the cells and the supernatant fluid was in turn assayed to determine the amount of residual free virus.

The difference in plaque counts between these two samples gave the number of infected cells.

All plaque assays were carried out in duplicate.

2) Infection in suspension

The nutrient medium was sucked off and the cell culture washed twice with PBS. Versene was then immediately added to the layer and a cell suspension obtained as above. The suspension was allowed to stand for two minutes to permit large clumps, if any, to sediment. The cells were transferred to a 16 ml. round bottom tube, centrifuged at 1200 rpm for two minutes, then resuspended in one ml. of a given virus inoculum. The cells were resuspended in the inoculum by vigorous pipetting with a .2 ml. pipette and incubated in a 37°C water bath for adsorption of the virus to take place. The cells were shaken frequently to prevent their clumping at the bottom of the tube. It was later found that infection

could be carried out in versene just as well as in PBS and without any clumping whatsoever. At the end of the adsorption period, the excess virus was washed off by repeated centrifugations. From then on, the steps were identical with those carried out in an experiment with infection on the plate.

One step growth curve:

One step growth curves (69) are obtained by preventing reinfection of susceptible cells by newly released virus. Reinfection can be prevented in cell suspensions by diluting the cells as soon as possible after infection and before the release of new virus begins.

Suspensions of infected cells in PBS were prepared as described above. In the early experiments such cells were diluted into NM I to give a final cell concentration of 2×10^4 cells per ml. This cell suspension was then placed in a well humidified incubator at $37^{\circ}C$ with a constant atmosphere of four per cent of CO_2 in air. After various time intervals, a volume of 1 ml. of the cell suspension was removed and centrifuged to sediment the cells. The supernate was then assayed for new virus.

It was later found that the ox serum present in the nutrient medium (NM I) had antiviral activity and that the virus grew as well without embryo extract. LAY was therefore used as the sole constituent of the growth medium.

Originally all the glassware used in an experiment was coated with paraffin. This precaution was later found to be superfluous.

Moreover, it was also found that the yield of progeny virus per infected cell is greatly increased by diluting the cells only to a final concentration of 2×10^5 cells per ml.

A typical growth curve of NDV is shown in Fig. 3 curve A.

EXPERIMENTAL RESULTS

I. Infection of host cells by NDV

A. Adsorption of active NDV to cellular monolayers

Since the knowledge of the adsorption coefficient of NDV is required in the interpretation of subsequent experimental results, the determination of this parameter appears to be a logical starting point for the present investigation.

Let us define as irreversibly adsorbed, particles which cannot be removed by repeated washings of the monolayer onto which they have adsorbed. Then, the number dV of particles which have adsorbed irreversibly from a suspension containing originally V_o virus particles during the time dt is given by the equation:

$$\frac{dV}{dt} = k C_o (V_o - V) \qquad (1)$$

where t is the time in minutes, k the adsorption coefficient in $cell^{-1}$ min^{-1} and C_o is the total number of cells in the host cell monolayer.

Integration of equation 1 from t=0 to t=T gives the number of particles irreversibly adsorbed at any time T, i.e.:

$$V = V_o (1 - e^{-k C_o t}) \qquad (2)$$

A plot of $\ln (1 - \frac{V}{V_o})$ - the natural logarithm of the fraction of non-adsorbed virus - versus the time t will then give a straight line with a slope equal to $(-k\ C_o)$.

The adsorption coefficient k was determined for B-NDV at 37°C in PBS according to equation 2 from the following experiment.

A volume of .5 ml. of a viral suspension containing approximately 200 pfu per ml. was plated onto each one of several fibroblast layers. After various time intervals ranging from 2 to 60 minutes, each one of a group of three infected layers was washed three times with warmed PBS, then overlaid with agar. The results are given in Table I and Fig. 1.

Table I

Time (min.)	Number of Plaques	Mean	Fraction Adsorbed
2	41,48,37	42	.39
4	44,40,41	42	.39
6	50,64,64	59	.55
8	78,84,75	79	.73
15	73,103,100	92	.85
30	105,110,98	101	1.00
45	110,106,105	107	1.00
60	128,96,100	108	1.00

From Fig. 1 it is seen that the non-adsorbed fraction of virus follows the kinetics expected from equation 2 when plotted on a semi-logarithmic scale as a function of the time.

The value for k was determined from the data of Table I by the method of Least Square. A value of .142 $min.^{-1}$ was obtained for k C_o the adsorption constant per monolayer.

The monolayer can be disrupted with versene and the number of single cells obtained is then determined by direct count. There are

approximately 2×10^7 cells per layer and k is then found to have a value of:

$$7.1 \times 10^{-9} \text{ cell}^{-1} \text{ minute}^{-1}$$

B. Infection of lung cells by active NDV

It is important for experimental purposes to know the rapidity with which the cells of a lung culture are infected under experimental conditions. Through most of this work, the interfering inoculum consisted of a volume of 1 ml. of a UVI virus suspension at a concentration of 5 to 10×10^8 particles per ml. From the adsorption constant of this virus determined above, it can be calculated (see Section VI B.) that with such an inoculum and under standard conditions 99 per cent of the susceptible cells should be infected within one minute or less. In order to check this point, the proportion of virus yielding cells (which shall hereinafter be referred to as yielders) was measured as a function of the time of adsorption of the viral inoculum in the following experiment.

Lung cells were infected on the plate with a volume of 1 ml. of an inoculum containing 8.2×10^8 pfu per ml. After a period of adsorption ranging from 2 to 30 minutes each layer was washed twice with warmed PBS. Anti-NDV serum at a dilution of 1:10 in PBS was then added for 15 minutes to neutralize the residual free virus. The number of yielders and the yield of new virus per yielder were determined as described under Methods. The results are given in Table II.

Table II

Adsorption time in minutes	2	4	6	10	15	20	30	Mean
Number of Yielders ($\times 10^{-5}$)	2.31	3.38	1.99	1.91	2.75	3.03	1.74	
Total number of cells ($\times 10^{-5}$)	9.1	12.0	5.8	7.0	9.8	11.0	7.8	
Fraction of Yielders	.254	.282	.343	.273	.281	.275	.223	.276
Virus production per Yielder	70	71	61	32	43	60	77	60

The data of Table II show that the number of yielders remains constant throughout the experiment. Thus, all the cells were infected within two minutes after the addition of the virus to the cells. This result was predictable on the basis of the previously determined rate of adsorption of the virus to the cell.

The fact that the fraction of infected cells is not equal to unity must be recorded. The reasons for this result are unknown. It is likely that the damage done to the infected cells in the course of the experiment is partly responsible for this effect. The ratio of yielders to total cells of about 30 per cent as given in Table II is an average value found in most of this work.

II. Effect of UV irradiation upon NDV

A. Loss of infectivity

It is well known that exposure of viruses to ultraviolet radiation results in the loss of their ability to produce progeny virus. Such particles are, however, far from being wholly inactive, as shown by their ability to kill cells onto which they adsorb, to interfere with the growth of superinfecting virus, to agglutinate red blood cells in the case of viruses of the mumps-influenza-NDV group and to fix complement (29). However, an increase in the dose of irradiation will also lead to the loss of these other viral properties in the order given above. The following experiments were therefore devised to find the UV dose with which NDV must be irradiated to lower the surviving fraction to an insignificant fraction yet without causing the loss of the interfering ability of the UVI virus.

One ml. samples of B-NDV in a suspension of 10 per cent ox serum in PBS containing 1.13×10^6 pfu were placed into small Petri dishes and irradiated for periods ranging from 15 to 120 seconds. Ox serum was used to protect this very unstable virus from thermal inactivation. The irradiated preparations were then tested for infectivity. The results are shown in Fig. 2 where the surviving fraction of the viral population is plotted on a semi-logarithmic scale as a function of the time of irradiation, i.e., the UV dose.

Fig. 2 shows that the loss of infectivity of B-NDV follows a single hit curve, i.e., it is exponential with no point of inflection at low doses. It also shows that under such conditions one hit

(equal to 37 per cent survival) occurs every 20 seconds.

In many subsequent experiments in which the stable K-NDV strain was used, ox serum was omitted. One hit occurred every twelve seconds when K-NDV was irradiated in PBS alone. The apparent difference in sensitivity between the two strains was caused by the opacity of the ox serum to UV light in the irradiation of B-NDV, as could be shown by measuring its absorbance to light of the wave length 254 mμ.

B. Adsorption of UVI-NDV

Since it is not possible to follow adsorption of UVI particles by the usual plaque technique, the adsorbability of UVI virus was studied by taking advantage of the fact that it still possesses the ability to hemagglutinate fowl erythrocytes. By necessity this test is not a sensitive one since small differences cannot be detected. It is adequate, however, to detect whether there is a great discrepancy between the rate of adsorption of the active virus and that of the UVI virus.

To carry out this test, the ability of the UVI virus to agglutinate red blood cells was tested first. Two 2 ml. samples were irradiated in the usual manner, one with an average of 15 hits per virus particle and the other with an average of 150 hits. The samples were then tested for their HA titers and compared with that of a control. In order to increase the accuracy of this test, two titrations were run in parallel on the same sample. The results are given in Table III.

Table III

Dilution factor of the UVI virus (HA titer)	80	160	60	120	240	Titer
Active	+	±	+	+	—	170
UV dose (15 hits)	+	±	+	+	—	170
UV dose (150 hits)	±	—	+	—	—	82

± is taken as the end point. When a + tube is followed by a — tube, the end point is taken as the geometic mean of the two.

The results show that irradiation with an average UV dose of 15 hits per particle did not decrease noticeably the ability of the virus to agglutinate erythrocytes. After exposure to 150 hits, however, 50 per cent of the HA capacity of the virus had been destroyed.

Adsorption of the UVI virus described above was measured in the following experiment. A volume of 1 ml. of UVI virus was added onto each one of several fibroblast layers. Several cultures were treated similarly with active virus. After 5, 10 or 20 minutes allowed for adsorption, the inocula were removed and tested for their HA ability. The results are given in Table IV.

Table IV

Adsorption time (min.)	0	5	10	20
HA titer in inoculum with active virus	170	140	120	80
HA titer in inoculum with UVI virus (15 hits)	170	140	110	70
HA titer in inoculum with UVI virus (150 hits)	82			49

It is evident from the data of Table IV that the UVI virus adsorbs at a rate not detectably different from that of active virus. The remaining hemagglutinin in the sample irradiated with an average of 150 hits per virus particle also adsorbs as well as does the active virus.

C. Enzymatic activity of UVI-NDV

Heat treatment of influenza and related viruses has shown that the enzymatic activity of the virus, i.e., its ability to destroy host cell receptors and elute from red blood cells, is destroyed before the adsorptive power.

It is of interest, therefore, for the interpretation of the phenomenon of interference, to test whether the enzymatic activity of UVI virus is destroyed although its adsorptive power is not. This was tested by determining the ability of the virus to adsorb onto and elute from fowl erythrocytes in the following experiment.

An exposure to an average UV irradiation of 15 hits per NDV particle was chosen as the inactivating dose.

A volume of .1 ml. UVI-NDV containing about 4×10^8 UVI particles (150 HA units) was mixed with .9 ml. of a one per cent red blood cell suspension. The virus was permitted to adsorb for 30 minutes at 4°C; elution does not occur at this temperature. The cells were finally resuspended in 1 ml. warmed PBS and incubated at 37°C for 30 minutes to allow elution of the virus to take place. The cells were again centrifuged down and the supernatant assayed for its HA titer. The control with active NDV at the same concentration was treated in an identical manner. Both tests were run in duplicate. The HA titers

of the final supernates are given in Table V.

Table V

Dilution of supernate	80	160	320	HA titer
Active NDV	+	±	−	160
Active NDV	+	±	−	160
UVI-NDV	+	±	−	160
UVI-NDV	+	±	−	160

Table V shows that NDV irradiated for three minutes adsorbs onto and elutes from red blood cells as well as does active NDV. It appears, therefore, that UVI-NDV retains its enzymatic activity.

III. Exclusion of fully infectious NDV by homologous UVI virus

A. Decrease in the fraction of yielders

UVI-NDV was tested for its ability to interfere with the growth of homologous, fully active virus in lung cells. The test was carried out as follows by using B-NDV.

Two lung cell monolayers were infected with .5 ml. of an inoculum irradiated with a dose of 12 hits and containing 4×10^8 UVI particles in NM I. The virus survival was 3×10^{-4}. A third culture received .5 ml. of NM I only. After an adsorption time of 30 minutes, the inoculum was removed and each culture washed twice with PBS. A volume of 5 ml. of NM I was then added to each plate which was incubated for an additional 30 minutes at 37°C. After that time interval, the nutrient medium was removed and the cultures were superinfected as

follows: One of the two cultures treated with UVI virus and the control received 1 ml. of a non-irradiated virus suspension containing 10^8 pfu, the remaining culture received 1 ml. of MM I only. The time interval between the two infections was 67 minutes. The superinfecting virus was adsorbed for 30 minutes after which time the number of yielders was determined. The results are given in Table VI.

Table VI

First Infection UVI	Second Infection active	Yielders per ml.	Total cells per ml.	Fraction of Yielders
+	−	10	2×10^4	negligible
+	+	8.7×10^2	2×10^4	4.3%
−	+	8.7×10^3	2×10^4	43%

The table shows that the fraction of yielders was 43% for the cells which received active virus alone, and only four per cent for the cells which had received UVI-NDV prior to their infection with active NDV.

B. All or none nature of the exclusion

The growth of NDV on lung cells may be described by several parameters, such as the length of the latent period, the length of the period of exponential increase, the slope of the growth curve in that period and the final yield of progeny virus per yielder. Although these characteristics vary according to the conditions of the experiment, they are relatively constant under a given set of conditions. Therefore, a logical start in the attempt at clarifying the problem of interference was to study the disturbing effects this phenomenon could

have upon the above mentioned parameters of the one step growth curve. This was done in the following manner.

The infected cells of the experiment described in Section III A. were diluted to 2 x 10^4 cells per ml. in NM I and the release of progeny virus was studied over a period of 24 hours. The results are plotted in Fig. 3.

Curve A of Fig. 3 is a normal growth curve of NDV in lung cells. Curve B represents the production of new virus in cells which were infected first with UVI-NDV, then superinfected with active virus 67 minutes later. In the light of the results of section A, curve B is the growth curve of NDV in cells which escaped interference by the UVI virus. As can be seen from the figure, the two curves are nearly identical except for the different absolute values. A yield of 14 progeny particles per cell is reached in both cases within 12 hours.

These results indicate that interference is of an all or none nature, i.e., either exclusion is complete or the cells release their normal yield of progeny virus at the normal rate.

To confirm this point, another experiment was performed. The UVI virus was used at three different multiplicities and the multiplicity of the superinfecting, active virus was kept constant. If exclusion is an all or none phenomenon, a variation in the multiplicity of the interfering agent should be reflected merely as a variation in the proportion of cells capable of producing virus. The results of such an experiment are shown in Fig. 4. The similarity of the four curves confirms the conclusion reached above that interference is of an all or none nature.

In addition, the variation in the proportion of yielders is that expected from the various multiplicities of UVI virus used in this experiment (see V A.).

C. Identification of the UVI particle as the interfering agent

It seems definite from the work done in the past with various "inactive" viruses as interfering agents that the interfering property is closely linked with the virus particle. Attempts to separate the interfering activity from the virus particles by biological, physical or chemical means have generally failed. Theidentification of the UVI-NDV particle is also shown unequivocally in this investigation. The results obtained in the preceding section point to a direct correlation between the multiplicity of the UVI virus and the amount of interference obtained. Moreover, as will be demonstrated in following sections, one UVI particle is sufficient to induce interference in the cell. In addition, the following experiment was performed to further link the interfering ability with the UVI-NDV particle. This experiment shows that the sedimentability of the interfering entity is similar to that of the virus particle and that the interfering entity is neutralized by anti-NDV serum.

The supernatant fluid of lung cultures was collected 24 hours after infection of the cells with K-NDV. This infectious suspension of NDV in LA was spun at 3000 rpm for six minutes in a clinical centrifuge to remove large cell debris. The supernate was then centrifuged with an average force of 45,000 times gravity in a Spinco model L ultracentrifuge to sediment the virus. The resulting pellet was resuspended in PBS; the activity of this viral suspension was 1.6×10^{10} pfu.

This viral stock solution was diluted eight-fold in PBS, irradiated with a UV dose of 15 hits on the average and centrifuged a second time at 45,000 g. The supernate of the second high speed centrifugation was saved to test its interfering power; the virus was again resuspended in PBS. A volume of .2 ml. of undiluted anti-NDV serum was added to 1.8 ml. of this last UVI virus suspension. The mixture was incubated at 37°C for 15 minutes. A volume of 2 ml. of the UVI virus suspension without antiserum was also incubated at 37°C for 15 minutes. After that incubation period, both suspensions along with the supernate of the second Spinco run, were tested for their interfering power.

Lung cells were treated on the plate with a volume of 1 ml. from each suspension; a fourth lung culture received PBS only. After an adsorption period of 10 minutes, all layers were washed three times with PBS, then superinfected with 1 ml. of an inoculum containing 1.6×10^9 active particles. The second inoculum was left on the plate for ten minutes; then the number of yielders in each sample was determined. The results are shown in Table VII.

Table VII

	Yielders per ml.	Total Cells per ml.	Fraction of Yielders
Second high speed supernate followed by active NDV	1.70×10^5	5.8×10^5	29.5%
Sedimented UVI virus treated with antiserum and followed by active NDV	1.51×10^5	3.4×10^5	44.5%
Sedimented UVI virus followed by active NDV	5.6×10^4	6.2×10^5	9.0%
Active NDV only	1.2×10^5	3.8×10^5	31.5%

The data of Table VII show that the supernate of the UVI virus suspension had no interfering power; neither did the UVI virus when treated with antiviral serum. The interfering ability was associated only with the unneutralized virus particle.

IV. Overcoming of interference

A. Nature of that fraction of the cells which escapes interference

In the results previously mentioned, one's attention is immediately drawn to that fraction of the cells which escapes interference by the UVI virus. That these infective centers consist of yielders can be demonstrated. The demonstration is based on the destruction of these infective centers by agents which destroy the cells but do not inactivate free virus. This was done in the following experiment.

A lung cell layer was infected with an input multiplicity of 100 UVI particles per lung cell. A second layer received PBS only. The inoculum was adsorbed for 30 minutes after which time it was removed and the lung cultures were washed twice with PBS. Each culture was then superinfected with an input multiplicity of 40 active NDV particles. Another period of 30 minutes was allowed for adsorption of the active virus. The number of yielders and the yield of progeny virus per yielder after 12 hours of incubation in LAY were then determined. Moreover, a sample of the cells from each culture was quickly frozen and thawed three times by placing their glass container alternately in a mixture of dry ice and 95 per cent ethyl alcohol and in a 37°C water bath. This procedure breaks open approximately 90 per cent or more of the cells but leaves the free virus undamaged (70). The results are given in Table VIII.

Table VIII

	UVI followed by active virus	Active virus alone
Yielders/ml.	1.6×10^4	1.7×10^5
Total cells/ml.	7.6×10^5	6.6×10^5
Fraction of yielders	2.0%	26%
Free virus/ml.	1.2×10^3	2.1×10^4
Yielders/ml. after freezing and thawing	1.8×10^3	1.9×10^4
Yield/yielder	31	53

In this experiment the fraction of cells which escaped interference consisted of eight per cent of the total number of infectable cells; 90 per cent of this fraction was eliminated by destruction of the cells. Moreover, the yield of new virus per yielder in that fraction was not significantly different from that in the control. This experiment, therefore, shows that exclusion is not induced in all the cells, some cells being superinfectable by active NDV after a primary infection with homologous UVI virus.

Subsequent experiments have also shown that the treatment of cells with anti-NDV serum after the addition of the superinfecting virus did not affect the size of this fraction resistant to interference, thus showing that this fraction is not constituted of free virus.

B. Reason for the lack of induction of exclusion in a fraction of the cells.

Certain cells could escape interference for the following reasons:

1. Insufficient amount of UVI virus to infect all the cells. This can be immediately ruled out because of the high multiplicities of infection with UVI virus which are more than sufficient to infect all the cells. (The determination of the multiplicities are done in section V.)

2. Physical blocking to infection by the UVI virus. In the lung cell layer, some cells might be inaccessible to the UVI particles because of their position. After the repeated washings to which the layer is subjected following the first infection, such cells could then become accessible to the superinfecting active virus. However, this possibility can also be ruled out by performing the infections in suspension where mechanical hindrance is at a minimum and all the cells have an equal chance of being infected. This was done in the following experiment.

Lung cells were infected in suspension as described under Methods with an input multiplicity of 400 UVI particles. Cells in a control received PBS only. After an adsorption period of 40 minutes, the cells were washed twice with PBS, then superinfected with an input multiplicity of 400 active NDV particles. An aliquot fraction of the cells which had received UVI virus were resuspended in sterile PBS. After a second adsorption period of 30 minutes, the number of infected cells was determined. The results are given in Table IX.

Table IX

	Yielders/ml.	Total cells/ml.	Fraction of yielders
UVI followed by active NDV	3.0×10^4	10×10^5	3.0%
Active NDV alone	1.2×10^6	11×10^5	100%
UVI-NDV alone	6.4×10^3	17×10^5	.4%

Table IX shows that although the fraction of cells where exclusion had not been induced had decreased to 3 per cent, it was, nevertheless, still a significant fraction of the cell population.

3. Inhomogeneity of the virus or of the host cells. Two reasons for inhomogeneity will be considered here. (a) The cell population could consist of two fractions: one being completely resistant to superinfection, the other fraction being completely susceptible to superinfection by active virus. (b) For each cell, there might exist a small probability that it can be superinfected by any given virus particle.

Let us now examine the predictions of these two hypotheses.

Hypothesis (a): From the Poisson probability function, the fraction of cells infected by the superinfecting virus is $(1-e^{-m})$ where m is the multiplicity of infection.

If there is a fraction f of the cells where exclusion occurs and a fraction (1-f) where it does not, the fraction of yielders $(\frac{C}{C_o})$ is:

$$\frac{C}{C_o} = (1-f\ )\ (1-e^{-m}) \qquad (1)$$

For large m, all the cells are superinfected and

$$\frac{C}{C_o} = (1-f\ ) = \text{a constant} \qquad (2)$$

Thus, the size of the fraction resistant to interference should be independent of m at high multiplicities under hypothesis (a).

Hypothesis (b): If there exists a small probability p for each superinfecting particle to multiply in any cell, the fraction of yielders will be:

$$\frac{C}{C_o} = (1-e^{-pm}) \qquad (3)$$

Thus, an increase in m will always be reflected by an increase in the fraction of yielders until it includes all the cells.

These two hypotheses were tested in the following experiments, where the multiplicity of superinfection was always more than sufficient to infect all the cells.

Three lung cultures were infected for 20 minutes with 1 ml. of inoculum containing 4×10^8 UVI particles (10 hits). A fourth plate received PBS only. All plates were washed twice and 5 ml. of MM I were added to each one for 10 minutes. 35 minutes after the first infection, the layers were superinfected with active NDV: one with 8×10^9 pfu, another with 4×10^9 pfu and the third one and the control received 8×10^8 pfu. The active inoculum was adsorbed for 20 minutes, then the

number of yielders in each culture was determined. The results are shown in Table X.

Table X

Experiment 1

Concentration of superinfecting virus	8×10^8	4×10^9	8×10^9	Control (no UVI)
Yielders	3.6%	12%	17%	44%

Experiment 2

Concentration of superinfecting virus	8×10^8	4×10^9	8×10^9	Control (no UVI)
Yielders	3.5%	6%	9%	42%

Table X, Experiment 1 shows a substantial rise in the fraction resistant to interference with the highest concentrations of superinfecting active virus.

This experiment was repeated by using a higher concentration of the interfering agent (8×10^9 UVI particles which received an average of 10 hits). Moreover, two ml. of anti-NDV serum (k = 30) at a dilution of 1/20 in PBS was added to each plate for 15 minutes after the second infection to remove as much of the free virus as possible. The results shown in Experiment 2, Table X again show a rise in the resistant fraction with a rise in the multiplicity of superinfection.

To ascertain that this rise in the number of yielders consists of cells actually infected and releasing virus, the following experiment was performed.

Four lung cultures were infected for 10 minutes with 1 ml. of

an inoculum containing 4×10^9 UVI particles (15 hits). A fifth culture received PBS only. All plates were then washed twice and superinfected with one ml. of active NDV at a concentration ranging from 8×10^8 to 1.6×10^{10} pfu per ml. The control received 8×10^8 active particles. The second adsorption period lasted 15 minutes and the plates were washed twice. A volume of two ml. of anti-NDV serum diluted 1:10 in PBS was added to each culture for 15 minutes to neutralize the free virus. The number of yielders in each culture and the yield of progeny virus per yielder were then determined. In addition, an aliquot sample from each culture was frozen and thawed three times, then assayed for its plaque forming ability. After such a treatment, the fraction of cells appearing as superinfected cells should disappear if it really consists of yielders. The results are given in Table XI.

Table XI

	Concentration of superinfecting active virus				
	8×10^8	1.6×10^9	8×10^9	1.6×10^{10}	Control (no UVI) 8×10^8
Yielders	3.9×10^4	1.0×10^5	1.7×10^5	1.6×10^5	2.7×10^5
Total cells per ml.	9.7×10^5	1.32×10^6	1.25×10^6	1.03×10^6	9.2×10^5
Fraction of Yielders	4.2%	7.9%	13.3%	15.3%	29.4%
Yielders as fraction of control	.14	.27	.45	.52	1.0
Free virus per ml.	1.3×10^3	1.8×10^3	6.3×10^3	1.2×10^4	9.7×10^3
Free virus plus yielders after freezing and thawing	3.7×10^3	3.7×10^3	1.4×10^4	3.0×10^4	3.5×10^4
Yield per Yielder	340	236	164	144	190

Table XI shows again a rise in the fraction of yielders with an increase in the multiplicity of the superinfecting virus. 90 per cent of this fraction is eliminated by destroying the cells. Moreover, the yield per yielder is not significantly different from that in the control. These results prove that these yielders were actually superinfected cells producing progeny virus like the controls.

As can be seen from the table, the fraction of yielders tends to approximately 50 per cent of the fraction obtained in the controls at the highest concentrations of superinfecting virus used. These results show, therefore, that neither hypothesis (a) nor hypothesis (b) is entirely correct. In 50 per cent of the cells, exclusion cannot be overcome; in the other 50 per cent, it is overcome according to hypothesis (b).

There is an analogy to this in the work with bacteriophage membranes which kill 10 to 30 per cent of the bacteria onto which they adsorb and induce a reversible exclusion in the others. Ghost preparations can be even obtained where up to 90 per cent of the membranes are killers (48).

Table XII shows the direct correlation between the increase in the concentration of the superinfecting virus and the rise in the number of cells superinfected after treatment with UVI virus. In calculating the data of Table XII, it is assumed that 50 per cent of the cells can be superinfected

Table XII

	Concentration of superinfecting virus			
	8×10^8	1.6×10^9	8.0×10^9	1.6×10^{10}
Yielders as fraction of the control	.28	.54	.90	1.0
Yielders expected from equation 3	.28	.48	.96	1.0

Table XII shows that the increase in the fraction of superinfected cells is nearly the increase expected from equation 3 above with an increase in the multiplicity of superinfection.

The probability that a cell can be superinfected (p of equation 4) is approximately four per cent in 50 per cent of the cells. The small probability of each superinfecting particle to induce a yielder can be explained in two different ways. Either there is an inhomogeneity (genetical or physiological) of the various particles, or the probability of a virus particle to produce a yielder varies with the conditions of its interaction with the cell. For instance, 50 per cent of the cells may have a part of their surface (4 per cent on the average) such that a superinfecting particle attaching there would be able to overcome interference.

A means is available to differentiate between a genetical and a physiological inhomogeneity of the virus population concerning the ability to overcome interference. The yield from cells in which interference is overcome should be tested for this ability. Preliminary experiments were carried out. They suggested that the overcoming of interference is not a genetical property of some virus particles.

V. Number of UVI particles necessary to obtain interference:

The number of UVI particles required to prevent the multiplication of active virus superinfecting the same cell was determined in two different manners.

A. Statistical proof that one UVI particle is sufficient.

The sole assumption required is that the virus particles (active or UVI) infect the cells in a random manner. If the multiplicity of infection of the UVI virus is defined as $m = \frac{V}{C_o}$ where V is the number of UVI particles having infected a system of C_o cells, the fraction of cells $\frac{C(r)}{C_o}$ infected by $\underline{r}$ particles is given by the Poisson distribution function as:

$$\frac{C(r)}{C_o} = \frac{m^r e^{-m}}{r!} \qquad (1)$$

If there is a critical number, say i+1, of UVI particles required to produce exclusion, the fraction of cells $\frac{C}{C_o}$ capable of being infected by superinfecting active virus is given by the expression:

$$\frac{C}{C_o} = e^{-m} \sum_{r=0}^{r=i} \frac{m^r}{r!} \qquad (2)$$

Now, if a single UVI particle is sufficient to induce exclusion, i = o and equation 2 becomes simply:

$$\frac{C}{C_o} = e^{-m} \qquad (3)$$

Similarly, if 2 UVI particles are required, we have i = 1 and:

$$\frac{C}{C_o} = e^{-m}(1+m) \qquad (4)$$

By plotting the experimental value of $\frac{C}{C_o}$ as a function of $\underline{m}$, it is possible to determine for which value of $\underline{i}$ this curve fits best equation 2. In Fig. 6 $\frac{C}{C_o}$ is plotted on a semilogarithmic scale as a function of $\underline{m}$ according to equations 3 and 4 for comparison with the

experimental findings.

To make such a comparison, m must be known as a function of the UVI virus concentration. This can be accomplished by determining the multiplicity of infection of the active virus before irradiation as a function of its concentration since adsorbability is not affected by UV treatment (see section II B.). In the following experiment the fraction of infected cells was determined at various active NDV concentrations.

Lung cells were infected on the plate with a volume of 1 ml. of an inoculum containing varying concentrations of NDV. The inoculum was adsorbed for 30 minutes and each plate was washed three times. The number of yielders was determined in the usual manner. The proportion of non-yielders was expected to decrease exponentially, as a function of m. The results are shown in Table XIII. The fraction of non-yielding cells is plotted on a semilogarithmic scale as a function of the virus concentration used in the inoculum, in Fig. 5. The straight line obtained can be extrapolated to the higher virus concentrations to get the corresponding multiplicities of infection.

Table XIII

Conc. of virus in inoculum	Fraction of maximal number of yielders	Multiplicity of infection
3.7×10^5	.01	.01
3.7×10^6	.095	.095
7.0×10^6	.14	.15
3.5×10^7	.57	.84
3.7×10^7	.57	.84
7×10^7	.64	1.03
3×10^8	1.0	-
5×10^8	1.0	-

The relationship between the interfering ability and the multiplicity of the UVI virus was then determined in the next experiments.

The general plan of the experiments was to infect a constant number of cells with varied amounts of UVI virus, then to superinfect these cells with a constant amount of active virus and to determine how many did not show interference.

Three experiments were carried out by infection on the plate. The UVI virus concentration was varied from 7×10^8 to 7×10^6 UVI particles per ml. The UVI inoculum was adsorbed for 30 minutes, then after washing the cultures, 5 ml. of NM I were added to each plate for 30 minutes. In the three experiments the concentration of the superinfecting active NDV was respectively: 5×10^7, 6.5×10^7 and 7.2×10^8 pfu per ml. The second inoculum was also adsorbed for 30 minutes, then the

number of yielders in each culture was determined. The results are given in Table XIV and the experimental values of $\frac{C}{C_o}$ are plotted as a function of m in Fig. 6.

Table XIV

	Conc. of UVI Virus	m	Fraction of Yielders	Fraction of Yielders Corrected for Overcoming of Interference	Yielders Fraction of Control
Expt. 1	7×10^8	16	1.4%	0	0
	1.4×10^8	3.2	5.0%	3.6%	.14
	7×10^7	1.6	7.0%	5.9%	.23
	2.3×10^7	.52	19.2%	18.6%	.71
	1.2×10^7	.27	21.5%	21%	.81
	7×10^6	.16	21.6	21.4%	.82
	Control		26%	26%	1.00
Expt. 2	2×10^8	4.5	4.2%	0	0
	1×10^8	2.3	7.8%	4.0%	.11
	5×10^7	1.1	10%	7.2%	.20
	Control		36%	36%	1.00
Expt. 3	7.2×10^7	1.64	8.3%	5.1%	.13
	3.6×10^7	.82	15.2%	13%	.34
	1.8×10^7	.41	21.7%	20%	.55
	9.0×10^6	.20	27.1%	26.4%	.67
	Control		38.1%	38.1%	1.00

Comparison of the experimental values of $\frac{C}{C_o}$ with the theoretical curves of Fig. 6 shows that they fit best the curve which requires that only one UVI particle be sufficient to produce interference.

B. Comparison of the multiplicity of exclusion with the multiplicity of infection.

To confirm in an independent manner the interpretation advanced in the preceding section, the following experiment was performed.

A fresh stock of NDV was prepared and used immediately to keep at a minimum the fraction of inactive particles. The multiplicity of infection at various input concentrations of active virus was then compared with the multiplicity of exclusion at identical input concentrations of UVI virus. The experiments were carried out with infection in suspension. The adsorption period of the inoculum lasted 30 minutes and anti-NDV serum diluted 1:10 in PBS was added to the cells for 15 minutes after the last infection. In the determination of the multiplicities of exclusion, the superinfecting active virus at a concentration of 2.4×10^9 pfu per ml. was added to the cells 50 minutes after the first infection with the UVI virus (15 hits). The number of yielders and the yield per yielder were determined as usual. The results are shown in Table XV a. and b.

Table XV a.

m of infection:

Conc. of active virus:	2.5×10^9	5.0×10^8	2.5×10^8	5.0×10^7	2.5×10^7
Yielders ($\times 10^{-5}$):	7.20	7.34	3.39	4.12	2.87
Total cells ($\times 10^{-5}$):	17.5	14.2	9.6	12.6	12.0
Fraction of Yielders:	.412	.516	.354	.327	.240
Yielders, Fraction of maximum:	1.0	1.0	.77	.70	.52
m (infection):			1.47	1.22	.73
Yield per Yielder:	55	68	78		

Table XV b.

m of exclusion:

Conc. of UVI virus:	2.5×10^9	5.0×10^8	2.5×10^8	5.0×10^7	control (no UVI)
Yielders ($\times 10^{-4}$):	3.17	3.6	5.2	12.4	33.4
Total cells ($\times 10^{-4}$):	73	77	70	88	87
Fraction of Yielders:	.044	.047	.074	.141	.387
Fraction of Yielders corrected for overcoming of interference:	0	0	.03	.10	.34
Yielders, Fraction of maximum:	0	0	.088	.295	1.0
m (exclusion)			2.43	1.22	
Yield per Yielder:	48	49	81	72	90

Table XV a. and b. shows that the multiplicity of exclusion for a given concentration of UVI virus is identical to the multiplicity of infection of active virus at the same concentration. Moreover, the plateaus giving 100 per cent yielders and 100 per cent interference are reached at the same concentration of active and UVI virus respectively. It is, therefore, concluded that the adsorption of one UVI particle on the lung cell is sufficient to inhibit the growth of the superinfecting virus.

VI. Speed of the interference reaction

Two independent types of experiments were performed in order to determine the speed at which exclusion is induced after the adsorption of the UVI particle.

A. Variation of the time interval between the two infections.

The previous experiments have shown that maximal exclusion is obtained when the time interval between infection by UVI virus and infection by active NDV is 30 minutes or longer. This time interval was, therefore, shortened in the following experiment.

An inoculum of one ml. containing 6×10^8 UVI particles (12 hits) was adsorbed for 10 minutes on lung cell layers. The cultures were washed twice with PBS and then superinfected with an inoculum containing 6×10^8 active particles after a time interval of $11\frac{1}{2}$, $15\frac{1}{2}$ or $21\frac{1}{2}$ minutes following the addition of the UVI virus to the layers. A control received active virus only. The fractions of yielders in each sample are given in Table XVI.

Table XVI

	Interval between infections			Control
	11½ min.	15½ min.	21½ min.	(no UVI)
Fraction of yielders	4.8%	2.1%	2.8%	30%
Fraction interfered with	.93	1.0	1.0	0

Table XVI shows that interference was already practically complete in all the cells when the time interval between the two infections was only 11½ minutes.

In order to shorten even further this time interval, infection was carried out on the monolayers with .8 ml. of an inoculum containing 2.8×10^9 UVI particles (10 hits) per ml. Under such conditions, 99 per cent of the cells are infected in .3 minutes. Then, without removing the first inoculum nor washing the cultures, .8 ml. of active virus at the same concentration was added to one of three plates after 1, 2 or 4 minutes respectively. After 30 minutes, the number of yielders was determined. A control received active virus only. The results are given in Table XVII.

Table XVII

	Interval between infections			Control
	1 min.	2 min.	4 min.	(no UVI)
Fraction of yielders	29.5%	28.8%	23%	39.5%
Fraction interfered with	.28	.30	.46	0

Table XVII shows that a considerable fraction of the cells, i.e. 28 per cent, can already exclude the superinfecting active virus when

the latter is added one minute after the UVI particles.

B. Determination of the speed of the reaction from the kinetics of infection.

The rapidity with which exclusion appears to be induced calls for more accurate means of determining the speed of the reaction than those described above. A study of the kinetics of infection of the cells by NDV was, therefore, attempted.

Theory: The number of virus particles V adsorbed at any time t from a suspension containing originally V_0 particles is given by equation 2 of Section I A. as:

$$V = V_0 (1 - e^{-k C_0 t}) \tag{1}$$

k is the adsorption constant of the virus which, as shown in Section II B., has the same value for both active and UVI particles. C_0 is the total number of cells in a lung culture.

From the Poisson probability function, the number of cells that have adsorbed virus at time t is:

$$C(t) = C_0 (1 - e^{-\frac{V}{C_0}}) \tag{2}$$

Substituting the value for V from equation 1, equation 2 becomes:

$$C = C_0 \left[1 - e^{-\frac{V_0}{C_0}(1 - e^{-k C_0 t})} \right] \tag{3}$$

The number of cells dC that adsorb virus for the first time during the interval dt is, therefore:

$$\frac{dC}{dt} = k C_0 V_0 e^{-k C_0 t} e^{-\frac{V_0}{C_0}(1 - e^{-k C_0 t})} \tag{4}$$

Let us now assume that it takes τ minutes for exclusion to be induced in a cell after the adsorption of one UVI particle. Or, more rigorously, τ is the minimal time interval following the adsorption of

the UVI virus after which the cell can no longer be superinfected by an active virus particle, in such a way as to become a yielder. This successful superinfection will be called simply "infection".

Let us, moreover, consider the case in which the UVI and the active particles are added simultaneously to the cells.

Then, for τ minutes, the infection of the host cells by the active virus will proceed unhindered by exclusion and at $t = \tau$ we will have $C(\tau)$ yielders:

$$C(\tau) = C_o \left[1 - e^{-\frac{V_a}{C_o}(1 - e^{-kC_o\tau})}\right] \tag{5}$$

where V_a is the initial concentration of active virus.

For any time greater than τ the number of cells, C_i $(t > \tau)$, where exclusion has been induced is:

$$C_i(t>\tau) = C_o \left| 1 - e^{-\frac{V_i}{C_o}\left[1 - e^{-kC_o(t-\tau)}\right]} \right| \tag{6}$$

where V_i is the initial concentration of UVI virus.

The number of cells still infectable is then:

$$1 - C_i(t>\tau) = C_o\, e^{-\frac{V_i}{C_o}\left[1 - e^{-kC_o(t-\tau)}\right]} \tag{7}$$

and from equations 4 and 7, the number of cells dC adsorbing active virus during dt becomes:

$$\frac{dC}{dt} = kC_oV_a e^{-kC_ot}\, e^{-\frac{V_a}{C_o}(1-e^{-kC_ot})} e^{-\frac{V_i}{C_o}\left[1-e^{-kC_o(t-\tau)}\right]} \tag{8}$$

The total number of yielders after a very long time t is then:

$$C_\infty = C(\tau) + \int_\tau^\infty kC_oV_a e^{-kC_ot} e^{-\frac{V_a}{C_o}(1-e^{-kC_ot})} e^{-\frac{V_i}{C_o}\left[1-e^{-kC_o(t-\tau)}\right]} dt \tag{9}$$

After integration $C(\tau)$ is replaced by its value given in equation 5:

$$C_\infty = C_o\left[1 - e^{-\frac{V_a}{C_o}(1-e^{-kC_o\tau})}\right] - C_o \frac{V_a e^{-\frac{V_a+V_i}{C_o}}}{V_a + V_i e^{kC_o\tau}} + C_o \frac{V_a e^{-\frac{V_a}{C_o}(1-e^{-kC_o\tau})}}{V_a + V_i e^{kC_o\tau}} \tag{10}$$

Since the experimental values of $\frac{V_a + V_i}{C_o}$ will always be very large, i.e. greater than 100, the second term on the right hand side of equation 10 is negligible. After a final rearrangement of the terms of equation 10, the total fraction of yielders $\frac{C_\infty}{C_o}$ is given by the following expression:

$$\frac{C_\infty}{C_o} = 1 - \frac{V_i e^{kC_o\tau}}{V_a + V_i e^{kC_o\tau}} \; e^{-\frac{V_a}{C_o}(1 - e^{-kC_o\tau})} \qquad (11)$$

Thence:

$$1 - \frac{C_\infty}{C_o} = \frac{V_i}{V_i + V_a e^{-kC_o\tau}} \; e^{-\frac{V_a}{C_o}(1 - e^{-kC_o\tau})} \qquad (12)$$

From the fraction ($\frac{C_\infty}{C_o}$) of yielders, it is now possible to determine τ as a function of V_a, the concentration of active virus in the inoculum.

Theoretical values of ($1 - \frac{C_\infty}{C_o}$), the fraction of non-yielders, were calculated according to equation 12 for values of τ ranging from .1 minute to 5 minutes. These values of ($1 - \frac{C_\infty}{C_o}$) are plotted on a semilogarithmic scale as a function of V_a in Figures 7 a and 7 b for comparison with the experimental values. The theoretical curves of Fig. 7 a are valid for V_i equal to 2×10^9 UVI particles per ml. of inoculum and the curves of Fig. 7 b for V_i equal to a concentration of 6×10^9 particles per ml. The experimental values of ($1 - \frac{C_\infty}{C_o}$) were determined under the conditions stipulated in deriving equation 12.

Several lung cell layers were infected with a volume of one ml. from one of several inocula containing a constant high concentration of UVI virus (V_i) and variable amounts of active virus (V_a). In the three experiments carried out, V_i was either 2×10^9 or 6×10^9 UVI

particles per ml. of inoculum and V_a was varied from 3.1×10^7 to 5×10^8 pfu per ml. The inoculum was adsorbed for 50 minutes, this time being considered infinite for all practical purposes; then the number of yielders ($\frac{C_\infty}{C_0}$) was determined for each culture. In the last experiment, the cells were treated with antiserum at the end of the adsorption period. Moreover, one layer was first infected with 2.5×10^9 UVI particles, then superinfected at a later time with 1.25×10^9 active particles to determine the size of that fraction of the cells where interference is overcome. This determination was necessary to make the correction noted in Section IV B. for the overcoming of exclusion in a fraction of the cells.

The results are presented in Table XVIII and plotted as a function of V_a in Figures 7 a and 7 b.

Table XVIII

Experiment 1 ($V_i = 6 \times 10^9$)

V_a ($\times 10^{-8}$)	4	2	1	Control (no UVI) 70
Yielders ($\times 10^{-5}$)	2.3	1.4	.81	6.2
Total cells ($\times 10^{-5}$)	10.4	13.5	10.8	8.2
C_∞/C_o	.31	.13	.10	1.0
$1-C_\infty/C_o$	.69	.87	.90	

Experiment 2 ($V_i = 2.26 \times 10^9$)

V_a ($\times 10^{-8}$)	2.8	1.9	1.1	Control (no UVI) 28
Yielders ($\times 10^{-5}$)	5.6	3.9	3.1	6.7
Total cells ($\times 10^{-5}$)	17.6	18.2	17.5	17.2
C_∞/C_o	.72	.46	.36	1.0
$1-C_\infty/C_o$	.28	.54	.64	

Experiment 3 ($V_i = 2 \times 10^9$)

V_a ($\times 10^{-8}$)	5	2.5	1.25	.625	.312	Control Active only	Control Overcoming of Exclusion
Yielders ($\times 10^{-5}$)	3.67	2.05	2.61	2.00	1.18	4.83	.60
Total cells ($\times 10^{-5}$)	11.1	8.3	13.4	10.7	9.5	10.5	12.0
C_∞/C_o	.61	.435	.32	.30	.20	1.0	0
$1-C_\infty/C_o$	.39	.565	.68	.70	.80		

Comparison of the experimental findings with the theoretical curves of Fig. 7 a shows that τ falls between .2 and 1.0 minute with a most probable value of .4 to .5 minute. In fig. 7 b, the experimental data show a good fit to the theoretical curve constructed with a value of .1 minute for τ. The curves of Fig. 7 a are valid when V_i is approximately equal to 2×10^9 UVI particles per ml. and the curves of Fig. 7 b for V_i equal to 6×10^9 UVI particles per ml. The differences in the value of τ suggest a dependence of τ on the multiplicity of the UVI virus. That this dependence actually exists is borne out by the following experiment, in which the concentration of active virus (V_a) was kept constant and the concentration of the UVI virus (V_i) was varied. V_a was equal to 3.1×10^7 pfu per ml. and V_i was varied from 1.4×10^8 to 3.5×10^7 UVI particles per ml. of inoculum. The general experimental procedure was identical to that employed in the determination of τ. The values of τ obtained by varying V_i are shown in Table XIX.

Table XIX

V_i	Yielders / Total cells	$\frac{C_\infty}{C_o}$	$1 - \frac{C_\infty}{C_o}$	τ in minutes	Multiplicity of UVI virus
1.4×10^8	10%	.35	.65	1.2	3.2
7.0×10^7	13.5%	.51	.49	1.9	1.6
3.5×10^7	18.5%	.82	.18	6.2	.8
none	22.5%	1.0			

τ was calculated from equation 12.

These results show that in Section V B., we were able to prove that one UVI particle is sufficient to bring about interference

because the time interval between the two infections - 60 or more minutes - was ample for interference to become established at any multiplicity of the UVI virus.

VII. Addition of UVI virus after the active one

The next series of experiments was designed to test whether UVI virus added after the active virus influences the growth of the latter as had been reported by Henle et al (60).

Two lung cultures were infected on the plate with one ml. of inoculum containing 7×10^9 pfu of active NDV. A third plate was infected with 7×10^9 UVI particles irradiated with an average of 15 hits. The inocula were adsorbed for 30 minutes; each plate was then washed twice with warm PBS and received 5 ml. of nutrient medium NM I. Thirty minutes later, the nutrient medium was removed and all plates were washed once with PBS. One of the two cultures previously infected with active virus was then superinfected with 7×10^9 UVI particles, the other one received PBS only. The third plate which had received UVI virus was superinfected with 7×10^9 active particles. The time interval between the two infections was one hour and twelve minutes. The second inoculum was adsorbed for 30 minutes then the number of yielders in each culture was determined. Single step growth curves were run on all three samples by diluting the infected cells 1:50 in NM I and incubating them as described in Methods.

The fractions of yielders in the interference control, the normal control and the test sample were respectively: .10, .76 and .57 of the total number of cells.

The growth curves shown in Fig. 8 indicate that the UVI

particles did not influence significantly the liberation of progeny virus when they were added 72 minutes after the addition of active NDV.

A second experiment was performed in which the time interval between the two infections was shortened. The inoculum was adsorbed for only ten minutes. Two test samples were run. To one culture, the UVI virus at a concentration of 1.25×10^9 particles per ml. (15 hits per particle on the average) was added 16 minutes after the active virus (1.25×10^9 pfu per ml.). In the second culture the UVI virus was added 36 minutes after the active virus. The number of yielders and the yield per yielder are given in Table XX, Experiment 1.

Table XX

Experiment 1

Time interval between infections:	36 min.	16 min.	Control (active virus alone)	Control (Interference)
Fraction of yielders :	49.5%	32.5%	45.5%	4.5%
Yield per yielder :	66	133	35	33

Experiment 2

Time interval between infections:	8 min.	8 min	Controls (active virus alone)	
Fraction of yielders :	20%	18%	22.5%	21.5%
Yield per yielder :	220	306	271	265

The results of experiment 1 again show that the UVI particles did not interfere when added 16 minutes after the active virus.

Nevertheless, a third experiment was carried out in which the time interval between the two infections was further shortened to only 8 minutes.

Four lung layers were infected for 5 minutes with 2.4×10^9 active NDV particles. The inocula were removed and the layers washed three times with PBS. Two layers were then superinfected with 4.8×10^9 UVI particles (15 hits) each. The other two layers received PBS only. The second inoculum was adsorbed for 5 minutes, then the plates were again washed three times with PBS. A volume of 2 ml. of anti-NDV serum diluted 1:10 in PBS was then added to all plates for 15 minutes. The fraction of yielders and their yields of new virus after 12 hours of incubation are given in Table XX, Experiment 2.

The results of Experiment 2 again show that the UVI virus had no effect upon the infected cells when added as soon as 8 minutes after the active virus.

It is, therefore, concluded that the UVI virus has no effect when it is added to the cells after the infectious particles.

VIII. Duration of exclusion

The next problem which presents itself is whether lung cells treated with UVI virus remain resistant to superinfection over an extended time period. This question can be answered simply by increasing the time interval between the addition of the interfering agent and superinfection with active virus.

1. The cells obtained from 8 lung layers were infected in suspension in a volume of 2 ml. of UVI virus containing 10^{10} particles

which had received an average of 10 hits. The cells from two more cultures were suspended in PBS only. After an adsorption period of 15 minutes, the excess virus was washed off by centrifugation of the cells. The cells infected with UVI virus were divided into two parts - half of them and the control cells were resuspended in LAY and incubated at 37°C, the second half of these cells was further subdivided into two parts. One half was superinfected with 2.5×10^9 active NDV, the other half received PBS only. The time interval between the two infections was 40 minutes. The fractions of infected cells were then suspended in 1 ml. of anti-NDV serum diluted 1: 10 for 10 minutes. The fractions of infected cells were then determined and are given in Table XXl.

After a time lapse of 141 minutes beyond their treatment with UVI virus, the remaining half of the cells was also subdivided into two fractions and treated as the first half had been. The control cells were also infected with 2.5×10^9 active NDV particles and treated likewise. The results are shown in Table XXl.

Table XXl.

Interval between infections:	Cells infected with:	Yielders/total cells:
40 minutes	UVI followed by active	.34%
	UVI alone	.06%
141 minutes	UVI followed by active	.57%
	UVI alone	.01%
	Active alone	21.0 %

The data of Table XXl show that exclusion was still present when the interval between the two infections was 141 minutes.

2. In order to study the duration of exclusion over a longer time period, the following experiment was performed.

Two lung cell layers were infected on the plate for one

hour with 4 x 10^9 UVI particles which had received an average UV dose of 15 hits. After removal of the inoculum, each plate was washed twice with PBS. A volume of 5 ml. of LAY was added to each culture which was then incubated at 37°C. The nutrient medium was removed 13 hours after the primary infection and .5 ml. of an inoculum containing 4 x 10^9 active particles per ml. was added to one of the layers. The other layer received sterile PBS. A control culture which had been treated in an identical manner but for the omission of UVI virus received also .5 ml. of an inoculum containing 4 x 10^9 pfu per ml. The second inoculum was adsorbed for 40 minutes and all the plates were washed three times with PBS. LAY was again added to all the layers which were then incubated at 37°C. After various time intervals, samples of the growth fluids were removed and assayed for progeny virus. The release of progeny virus from these three cultures is shown in Fig. 9.

Fig. 9 shows that the cells which had been infected with UVI virus 13 hours prior to their infection with fully infectious virus released 70 times less progeny virus than the cells in the control. Therefore, exclusion was still present 13 hours after infection of the cells with UVI virus.

In addition to the above, the following information was obtained from a microscopic examination of the infected cell layers.

The lung cultures which had been treated with UVI virus prior to their infection with active NDV were still in excellent condition 26 hours after the second infection. On the other hand, many necrotic areas had appeared in the layers which received only active NDV. However, 53 hours after the second infection, small necrotic areas had appeared in the cultures which had received only UVI virus. These necrotic foci were present in a greater number in the layers which had received active virus

after the UVI particles. The cells of the cultures which had received only active NDV were completely destroyed after 53 hours.

It appears, that the resistance to infection had disappeared in a good many cells 66 hours after their infection with UVI virus. Therefore resistance to infection lasts at least 26 hours but less than 60 hours.

IX. Noninfectious hemagglutinin (NIH)

NIH is constituted of particles lacking infectivity but still capable of hemagglutinating erythrocytes. The presence of those particles in a virus preparation is indicated by a decrease in the ratio of infectivity to HA titer.

It must be recalled that NIH of influenza virus is thought to be either an intermediate in the process of virus synthesis, a by-product of virus synthesis or a breakdown product of newly formed virus which was inactivated after its release (49, 71, 72, 73). In the case of NDV, Granoff et al (74) (1950) have reported two components, one associated with the infectious principle, the other distinctly smaller and non-infectious which these authors interpreted as a precursor of the fully active particle. No discussion of this highly controversial subject will be attempted.

It was considered interesting to determine whether any incomplete form of NDV capable of hemagglutinating red blood cells was produced by those cells which did not release any fully infectious particles because of interference. This was done in the following manner.

HA titrations were run on the supernatant growth medium of the three cultures used in Experiment VIII-2 above ten hours after their infection with active virus. At that time, the plaque forming titers of new

virus were: 1.5×10^6, 8×10^6 and 2×10^9 pfu per ml. respectively for the cells which had received UVI virus only, UVI virus followed by active virus 13 hours later and active virus only (see Fig. 9). The corresponding HA titers obtained were: less than 10, 10 and 640.

The two significant HA values give a ratio of pfu to HA titer well representative of a suspension consisting entirely of fully active particles, i.e. greater than 10^6. The same results were obtained in one other experiment.

We may, therefore, conclude that no abnormal amount of NIH is produced in cells where interference occurs.

X. Destruction of the interfering activity

A. UV dose

NDV was subjected to doses of UV radiation much higher than those previously used to see whether there would be an accompanying decrease in the ability of the UVI virus to cause interference.

1. Specimens of K-NDV containing 3×10^8 particles per ml. were irradiated as described under Methods. Each sample received an average of 10, 50 or 100 hits.

Lung cultures were infected on the plate simultaneously with UVI virus from one or the other of the three irradiated suspensions and with active NDV. The inoculum (1 ml.) contained 2.83×10^8 UVI particles and 1.41×10^8 active particles. One cell layer received active virus only. The inoculum was adsorbed for 50 minutes, then the fraction of infected cells in each culture was determined. Such an experimental procedure permitted a calculation of τ according to equation 12 of section VI-B.

The results are given in Table XXII.

Table XXII

	10 hits	50 hits	100 hits	Control (no UVI)
Yielders ($\times 10^{-5}$)	2.38	3.09	2.2	3.84
Total cells ($\times 10^{-5}$)	9.0	10.9	9.1	8.6
Fraction of yielders	.264	.283	.242	.446

The data of Table XXII show that NDV irradiated with as many as 100 hits on the average is just as good an interfering agent as virus irradiated with 10 hits only. From the fractions of infected cells, $\underline{\tau}$ was determined according to equation 12 of Section VI-B. It had a value of .4 minutes with all three UVI virus preparations.

2. A second experiment was carried out with K-NDV irradiated with an average of 15, 100, or 150 hits per particle. In this case, the UVI virus in 1 ml. of inoculum containing 3×10^{8} UVI particles was added to the cell layers 37 minutes prior to infection with active NDV at the same concentration. In both infections, the adsorption time for the inoculum was 30 minutes. The fractions of yielders and the virus produced after twelve hours per yielder were determined and are given in Table XXIII.

Table XXIII

	15 hits	100 hits	150 hits	Control (no UVI)
Yielders ($\times 10^{-4}$)	6.2	5.6	6.24	15.8
Total cells ($\times 10^{-4}$)	72	82	67	50
Fraction of yielders	.086	.068	.093	.32
Yield per yielder	105	79	81	78

The data of Table XXIII show no apparent loss of interfering ability by the virus after it has been irradiated with UV light with

an average of 150 hits per particle.

This apparent discrepancy with the results of Section II-B, where it was found that 50 per cent of the UVI virus loses its ability to agglutinate red cells after such a dose of irradiation, can be easily explained. In the present experiment, the multiplicity of infection of the UVI virus was so high, i.e. 6.8, that a 50 per-cent decrease in the effective multiplicity of the interfering virus could not have been detected. Indeed, with a multiplicity of 3.4, only 3.3 per cent of the host cells would not be infected by UVI virus and this fraction is obscured by those cells in which inter-ference is overcome.

From this experiment we may conclude that the interfering activity of NDV appears much more resistant to UV irradiation than that of the influenza viruses. In fact, it has been reported that after being subjected to an average of 150 hits by UV irradiation, PR-8 influenza A virus retained only 1 per cent of its interfering ability and Lee influenza B only 2 per cent (29).

B. Effect of anti-NDV serum

The addition of anti-NDV serum to the lung cells after in-fection with UVI virus but before addition of fully active virus had an unexpected effect since it removed interference in a considerable fraction of the cells.

1. Two lung cultures were infected with 1.47×10^9 UVI particles. A third culture received PBS only. The inoculum was adsorbed for 20 minutes, then each layer was washed twice with PBS. A volume of two ml. of anti-NDV serum diluted 1:10 in PBS was added for 15 minutes to one culture 25 minutes after the latter had been infected with UVI

virus. Antiserum was also added to the control layer. The other culture received PBS only. Under these conditions, the serum neutralizes free virus to 4 x 10^{-4} survivors. The rabbit serum had no anti-cell activity since it had been adsorbed with chicken embryonic cells.

After the removal of the antiserum, the layers were thoroughly freed of any residual antiviral activity by three consecutive washings with PBS. They were then superinfected with 1.47 x 10^9 active NDV particles. The second inoculum was adsorbed for 20 minutes, then the fractions of yielders were determined. The results are given in Table XXIV.

Table XXIV

Cells treated with:	Yielders per ml.	Total cells per ml.(x 10^{-4})	Fraction of Yielders
UVI virus, anti-NDV serum, active virus:	4.9 x 10^5	130	.385
UVI virus, active virus:	6.0 x 10^4	91	.066
Anti-NDV serum, active virus:	4.8 x 10^5	85	.564

The data of Table XXIV show that the addition of anti-NDV serum 25 minutes after the addition of UVI virus destroyed interference in 57 per cent of the cells. It should be mentioned that this inhibition is not caused by an inhibition of the adsorption of the UVI particles, since with 1.47 x 10^9 UVI particles in the inoculum 99 per cent of the host cells adsorb virus within .6 minute. The antiserum was, therefore, able to inhibit the expression of exclusion after the UVI virus had adsorbed onto the lung cell.

2. The experiment was repeated with the following modifications. As a control, normal rabbit serum was added to a lung culture 25 minutes after the addition of UVI virus and all the layers were treated with anti-NDV serum after superinfection with active virus to remove the free virus. Moreover, the second inoculum contained twice as many active particles, i.e., 2.94×10^9 pfu. The results are shown in Table XXV.

Table XXV

Cells treated with:	Yielders per ml.	Total cells per ml. ($\times 10^{-4}$)	Fraction of yielders
UVI virus, anti-NDV serum, active virus, anti-NDV serum:	5.4×10^4	58	.0935
UVI virus, normal serum, active virus, anti-NDV serum:	1.0×10^4	53	.020
Anti-NDV serum, active virus, anti-NDV serum:	1.05×10^5	66	.155

The data of Table XXV show that normal serum did not inhibit the expression of exclusion, whereas anti-NDV serum did in 47.5 per cent of the cells.

This inhibition of interference appears, therefore, to be caused by the specific action of the antibodies upon the adsorbed virus particles.

3. A second series of experiments was performed to study the effect upon exclusion of the variation in the time interval between infection with UVI virus and treatment with antiserum.

Lung cultures were infected with 1.4×10^9 UVI particles for 10 minutes. After various time intervals, ranging from $13\frac{1}{2}$ to 90

minutes following the addition of the UVI virus, a volume of two ml. of anti-NDV serum (1:10 in PBS) was added to the layer for 15 minutes. All the antiviral neutralizing ability was removed by three consecutive washings in PBS. The cells were then superinfected with 1.4×10^9 active particles for 10 minutes. After the second infection, the cultures were again treated with antiserum to neutralize the free virus. A control was run without adding antiserum after infection with the UVI virus. A second control did not receive UVI virus. The controls were washed with PBS in a manner identical to that of the test samples. Two such experiments were carried out. The number of yielders and the yield of virus per yielder are shown in Table XXVI.

Table XXVI

Experiment 1

Time interval between UVI virus and serum:	Controls: no serum	Controls: No UVI	13½ min.	28 min.	44 min.
Fraction of yielders to total cells:	1.3%	21%	19.5%	20%	10.5%
Yield per yielder	6	38	27	61	36
Fraction of yielders to control yielders:	6 %	100%	100%	100%	50%

Experiment 2

Time interval between UVI virus and serum:	Controls: no serum	Controls: No UVI	30 min.	60 min.	90 min.
Fraction of yielders to total cells:	31%	33%	18.2%	16.6%	16.5%
Yield per yielder:	31	105	36	49	92
Fraction of yielders to control yielders:	9.4%	100%	55%	50%	50%

The data of Table XXVI show that the addition of anti-NDV serum up to 28 minutes after the addition of the UVI virus, but before infection with active virus prevents the occurrence of exclusion. When the antiserum is added later - from 30 to 90 minutes - only 59 per cent of the susceptible cells are superinfected.

4. In the light of these results, it was necessary to reinvestigate the effect of anti-NDV serum upon normally infected cells, i.e., cells which did not receive UVI virus prior to active virus.

Two experiments were performed in which anti-NDV serum (1:10 in PBS) was added for 15 minutes to cells at various time intervals after the addition of fully active NDV. The infection was carried out on the monolayer. In the first experiment, the inoculum containing 2.5×10^9 pfu was adsorbed for 10 minutes. In the second experiment, the inoculum contained 5×10^9 pfu and was adsorbed for only 5 minutes. In both experiments a control was treated with normal rabbit serum diluted 1:10 in PBS. In the second experiment the infected cells were frozen and thawed three times. The number of yielders and the yield per yielder were determined. The results are given in Table XXVII.

Table XXVII

Experiment 1

Antiserum added after:	13 min.	28 min.	43 min.	Normal serum control
Yielders (x 10^{-5})/ml.:	1.81	1.73	1.6	1.54
Total cells (x 10^{-5})/ml.:	8.1	8.1	8.5	5.1
Fraction of yielders:	.224	.215	.190	.305
Yield/yielder:	23	41	19	43

Experiment 2

Antiserum added after:	7½ min.	11 min.	15½ min.	19½ min.	Normal serum control
Yielders (x 10^{-5})/ml:	3.31	1.87	1.88	3.85	4.0
Total cells(x 10^{-5})/ml.	14.0	8.4	9.0	14.0	15.1
Fraction of yielders:	.237	.223	.210	.275	.265
Yield/yielder:	62	83	73	39	44
Free virus/ml.(x 10^{-5}):	.15	.16	.12	.15	.82
Yielders after freezing and thawing (x 10^{-5}):	.52	.19	.21	.33	0

The data of Table XXVII show that anti-NDV serum added to lung cells as soon as 7½ minutes after the addition of active NDV does not decrease significantly the number of yielders nor the yield of progeny virus.

Through the effect of anti-NDV serum, the first difference between UVI and fully infectious NDV has been detected in the sequence of events following adsorption of the virus particle to the lung cell. The interfering ability of the UVI particle appears to remain sensitive

to antibodies indefinitely, at least in 50 per cent of the cells, whereas the infecting ability of the active particle cannot be inhibited after seven minutes at most.

It is very tempting to identify the fraction of cells - 50 per cent - which cannot be superinfected when the antiserum is added 30 or more minutes after the UVI virus, with the fraction of cells - also 50 per cent - in which exclusion cannot be overcome by high multiplicity of the superinfecting virus, as reported in Section IV-B-5. This fraction could consist of killed cells. In the surviving cells, exclusion would then be eliminated either by neutralizing the UVI virus with specific antiserum or by overcoming exclusion with a great number of superinfecting active particles. This would suggest that the UVI virus remains at all times susceptible to specific antibodies, namely on the cell surface. However, the possibility that the UVI virus causes in 50 per cent of the cells a different type of exclusion cannot be excluded.

5. It was of interest to test whether the superinfecting active particles remained susceptible to antibodies after they had adsorbed onto cells which had previously been treated with UVI virus.

Lung cells were infected on the plate with 2.7×10^9 UVI particles for five minutes. The layers were then superinfected with 1.6×10^9 active particles for another five minutes. Antiserum (1:10 in PBS) was added $19\frac{1}{2}$ minutes after the first infection.

After such a treatment, it was found that only 10 per cent of the cells were infected, whereas in the control 32 per cent of the

cells were infected. A control showed that in this experiment the antiserum removed the exclusion as usual.

Thus, the superinfecting virus was sensitive to antibody at the moment that the exclusion produced by the UVI virus was removed by the antibody.

This implies that the superinfecting active virus either became rapidly inactivated after its adsorption to the cells in which interference was established or was neutralized by the antiserum. If the second alternative is correct, the superinfecting active particles were prevented from penetrating into the cells, since under normal conditions active virus becomes insensitive to antibody less than seven minutes after its attachment to the host cell.

C. Effect of RDE

RDE is well known to cause the elution from erythrocytes of virus which has lost its enzymatic activity and to facilitate the elution of adsorbed active virus. This enzyme was, therefore, tested for its ability to destroy exclusion either by removal of the UVI particle or by some modifying action on the cell surface.

Six lung cultures were divided into two groups. The first group was infected with 4.4×10^8 UVI particles for 10 minutes, the second group received PBS only. Each layer was then washed twice with PBS. Fifteen minutes after the addition of UVI virus, one culture from each group was then treated with two ml. of anti-NDV serum (1:10), a second culture from each group received one ml. of an RDE suspension containing 800 units and the remaining cultures received only PBS.

This treatment lasted 30 minutes at 37°C. All cultures were then washed three times with PBS and incubated. After that time, all the layers were superinfected with 4.4×10^8 active NDV particles for 10 minutes. The layers were again washed twice with PBS, then treated with antiserum (1:10) for 15 minutes. The number of yielders in each culture was determined and is given in Table XXVIII.

Table XXVIII

Cells were treated in order with:

UVI Virus	Antiserum	RDE	Active Virus	Antiserum	Yielders	Total Cells ($\times 10^{-4}$)	Fraction of Yielders
+			+	+	8.1×10^4	120	.066
+	+		+	+	2.23×10^5	70	.320
+		+	+	+	4.80×10^4	122	.039
	+		+	+	3.2×10^5	140	.230
	+		+	+	3.03×10^5	92	.330
		+	+	+	2.55×10^5	75	.340

The data of Table XXVIII show that RDE did not inhibit the expression of exclusion whereas antiserum did so in 100 per cent of the cells.

This lack of effect by RDE was not surprising. In fact, NDV possesses a powerful enzyme system capable of destroying the corresponding cellular receptors and elutes from erythrocytes very rapidly. The UVI virus has not lost any of this activity, as shown in Section II-C.

D. Effect of trypsin.

Trypsin was used in an attempt at destroying the exclusion either by acting on the cell surface or by freeing the UVI particle from its site of attachment to the cell.

A cell suspension was prepared from nine lung cell cultures. Half of these cells were infected by resuspending them for 5 minutes into 3 ml. of inoculum containing 4.4×10^9 UVI particles; the remainder of the cells was resuspended in PBS only. After two washings and 27 minutes later aliquots from each cell suspension were treated as follows: some cells received PBS only, others anti-NDV serum (1:10 in PBS) and the remaining cells .05 per cent trypsin in PBS (all materials were prewarmed to 37°C). The cells were incubated at 37°C.for 10 minutes. After two washings, the cells were superinfected by resuspending them for 10 minutes into an active NDV suspension containing 1.1×10^9 particles per ml. (.5 ml. of inoculum per tube). The fraction of yielders was then determined for each sample. The results are given in Table XXIX.

Table XXIX

Cells treated with:				Fraction of Yielders
UVI Virus	Antiserum	Trypsin	Active Virus	
			+	90%
	+		+	100%
		+	+	100%
+			+	0
+	+		+	>70%
+		+	+	0

The data of Table XXIX show that trypsin did not inhibit the expression of exclusion when added 27 minutes after the UVI virus, whereas anti-NDV serum did.

XI. Disappearance of the superinfecting active virus

It was repeatedly noted in the course of these experiments that the amount of residual free virus in the supernatant of the last cell suspension (see Methods) was 3 to 140 times (20 times on the average) larger in control cell suspensions than in suspensions of cells which had been infected with UVI virus prior to the addition of active NDV. Moreover, the amount of active virus adsorbed from the inoculum by both types of cells was identical.

It appeared, therefore, as if after infection with UVI virus, the lung cells acquired the ability either to break down the superinfecting active particles or to prevent their elution. In an attempt at gaining more information about this phenomenon, the disappearance of

the residual free active virus was studied over the entire course of an experiment.

A lung cell layer was infected with 7.8×10^9 UVI particles for 40 minutes; a second layer received PBS only. After two washings both cultures were superinfected with 7.8×10^9 active NDV particles for 30 minutes. A volume of .8 ml. of the second inoculum was removed and each plate was then washed six times by the successive addition and removal of 20 ml. of prewarmed PBS. A sample from each one of those washings was saved for assay at a later time. A volume of 5 ml. of Versene was added to each culture for 5 minutes and after disruption of the cell layer, the cells were centifuged down. A sample of this supernatant was also saved for assay. The cells were resuspended in 5 ml. of PBS without Ca^{++} and Mg^{++} and sedimented again. This procedure was repeated twice and all the supernatants were again saved for free virus determinations. The cells were finally resuspended as follows: the cells which received only active virus in 2.5 ml. of PBS, the test cells in 1.5 ml. of PBS. The total number of cells in each suspension was respectively: 1.25 and 1.8×10^6. The number of infected cells and the amount of residual free virus in all the suspensions were determined as usual. The results are given in Table XXX.

To detect elution of the virus, a sample of the cells was incubated at 37°C. for 60 minutes. These cells were then sedimented by centrifugation and the amount of free virus in the supernatant was determined. Moreover, in an attempt to liberate and determine the amount of any bound virus, samples of the cells were frozen and thawed three times. This operation was performed twice, 44 minutes

after the last centrifugation and after one additional hour of incubation at 37°C. All the results are given in Table XXX. (Table XXX is on page 90)

The data of Table XXX can be analyzed as follows:

There is on the average twice as much free virus in the washings of the control than in the washings of cells infected with UVI virus prior to the addition of active virus. These washings were carried out while the cell layers were still intact on the plate. Upon treatment of these layers with Versene and their subsequent disruption, 4.3×10^7 active virus particles were freed from the control cells, but only 5.2×10^6 active particles were freed from the test cells. In the last supernatant of the cells there was 20 times more free virus than in the control. The missing virus was not recovered by breaking up the cells and no elution of virus took place upon incubating the cells for an extra hour at 37°C.

The disappearance of superinfecting active virus when cells are treated previously with UVI virus was thus confirmed. Whether the virus is broken down or remains adsorbed to cells thus treated could only be tested with radio-labelled virus; such a test is beyond the scope of the present work.

The debris of lung cells infected with UVI virus were tested for their ability to inactivate free virus. The cells from two lung cultures infected with UVI-NDV were broken up by freezing and thawing them three times in succession. The cells were resuspended in 1 ml.

of infective PBS containing 5 x 10^8 active particles and incubated at 37°C. for six hours. There was no drop in the titer of the virus suspension. Normal cells treated likewise gave the same results.

XII. Morphological changes of the cells infected with UVI virus

Macroscopic and microscopic examination of lung cell layers infected with UVI-NDV did not reveal any morphological change. These cells took up the vital stain, neutral red, as well as did normal cells. Moreover, when the progeny of the surviving fraction of the UVI virus population was kept negligible with antiserum in the growth medium, the lung layers remained intact for seven days, their normal life span; on the contrary, cells infected by active virus can be clearly recognized as dead 24 hours after infection. However, the morphological observation would not detect the death of 50 per cent of the cells, which may occur as mentioned in Sections IV-B-3 and X-B. Whether the cells infected by UVI virus remain viable indefinitely will have to be tested in the future by studying their ability to multiply and produce colonies.

Table XXX

Free virus in washings of the intact layers:

	#1	#2	#3	#4	#5	#6
Control	9.2×10^8	1.8×10^7	6×10^6	7.4×10^5	4.6×10^5	2×10^5
Test	4.2×10^8	1.5×10^7	1.6×10^6	4×10^5	1.6×10^5	1×10^5

Free virus in supernatants of cells after disruption of the layers:

	Versene	First Centrif.	Second Centrif.	Third Centrif.	Third Centrifugation Delayed 1 Hour
Control	4.3×10^7	1.42×10^6	6.25×10^5	3.52×10^5	2.63×10^5
Test	5.2×10^6	9.0×10^5	2.0×10^4	1.8×10^4	1.72×10^4

Infective centers = infected cells + free virus in last supernate:

	Infective Centers	Yielders	Total Cells	Fraction of Yielders	After freezing and thawing: Infective Centers	After freezing and thawing: Inf. Centers After 1 hr. Incubation
Control	1.71×10^6	1.36×10^6	2.5×10^6	54 %	2.75×10^5	2.3×10^5
Test	1.20×10^5	1.02×10^5	2.1×10^6	4.8 %	1.86×10^4	1.9×10^4

DISCUSSION AND CONCLUSIONS

The results of this investigation will be briefly reviewed and their implications will be brought out.

A. Effect of UV light upon the virus.

NDV irradiated with UV light loses its infectivity, i.e. its ability to give rise to progeny virus. This inactivation follows the kinetics of a single hit process: damage to one critical site is sufficient to render the virus non-infectious. NDV rendered non-infectious in this manner (UVI virus) adsorbs to the host cells at the same rate as does fully infectious virus. The adsorption rate coefficient is 7.1×10^{-9} $cell^{-1}$ $min.^{-1}$ under the current experimental conditions. The UVI virus exposed to 15 hits, also retains its ability to adsorb to and elute from erythrocytes as well as the fully active virus does: therefore the enzymatic activity of NDV, which is responsible for the destruction of the cellular receptors, remains undamaged after this treatment.

The interfering ability of the virus is resistant to large doses of UV irradiation (100 hits or more) and appears to be lost only when the UVI particle is unable to adsorb to the cell.

It is not known whether the persistence of the enzymatic activity is required for the interfering ability of the virus. The present experiments do not give sufficient information on this point, since the survival of the enzymatic activity as a function of the UV dose was not determined. However, some information on this point can be deduced by the fact that NDV heated at $56^{\circ}C$ for one hour does not interfere. Heated virus has presumably lost its enzymatic activity

since it can still adsorb to red blood cells but cannot elute spontaneously (see Introduction). These findings may suggest that adsorption to the host cell is not sufficient to bring about exclusion and that persistence of the enzymatic activity may be essential. It must be kept in mind, however, that virus particles heated at 56°C for one hour sustain also other kinds of damage beside the destruction of the enzymatic system under discussion. UVI virus also loses its interfering ability upon heating. A study of the interfering ability of UVI virus particles which have been damaged by graded doses of agents, such as heat, UV light or enzymes, should clarify this point; such an analysis would also allow a study of the process of penetration of the virus into the cell, particularly if virus labelled with radioactive tracers were used.

B. Quantitative aspects of exclusion

The interfering activity is a property of the virus particle, which is expressed upon its attachment to the cell: in fact, the activity is present only in UV irradiated suspensions of NDV, not in the virus-free supernates of such suspensions, nor in suspensions where the UVI virus has been neutralized with specific antiserum. In the system used (UVI virus, chicken embryo lung cells) one UVI particle attached to a cell is sufficient to inhibit the production of progeny virus when the cell is superinfected by active virus. However, interference is not established immediately as will be indicated later.

Interference is of an all-or-none nature: either exclusion is complete or the cells release their normal yield of progeny virus at

the normal rate. There is no production of non-infectious hemagglutinin in cells where interference occurs. Virus production is, therefore blocked at a relatively early step.

Interference is not established instantaneously when the cells are mixed with the UVI virus. There is a maximal time interval after infection by UVI virus during which the cell can still be superinfected by active virus. This time interval is a function of the multiplicity of infection of the UVI virus; it varies from a maximum of approximately six minutes with one UVI particle to .1 minute or less with a multiplicity of 140. The existence of this time lag can be explained in two different ways. Either exclusion is induced during that time interval, or the UVI particle must have that much head-start on the active virus and eventually blocks the path of virus synthesis. An increase in the multiplicity of the UVI virus is reflected by an increase in the speed at which the interfering reaction takes place. The meaning of this cumulative effect of several UVI particles - one being however sufficient - will be discussed later.

The UVI virus has no effect when it is added to the cells after their infection with fully active virus. This finding agrees with the hypothesis that exclusion affects the early phase of virus penetration.

The resistance to superinfection produced by the UVI virus lasts at least for 26 hours but less than 60 hours (at least in a fraction of the cells) after the addition of UVI virus to the lung cells. This may be the time interval required by the cell to

regenerate certain cellular components or for the UVI virus to lose its interfering effect either by destruction or elution.

It would be of great interest to test what effect a second infection with UVI virus, at various time intervals after the first one, would have upon the duration of the induced resistance to infection. For instance: a lack of effect, i.e. no increase in the duration, might imply that there is also exclusion of the superinfecting UVI virus - the critical sites themselves might be blocked - whereas an increase in the duration of resistance could mean that the loss of exclusion is caused by elution of the UVI particles.

C. Overcoming of exclusion

Occurrence or non-occurrence of exclusion depends on the multiplicity of the superinfecting virus in 50 per cent of the cells. There exists for each one of these cells a small finite probability that a superinfecting active virus particle will be successful in initiating infection. This phenomenon may be brought about either by the presence in the virus population of a small fraction which for genetic or physiological reasons is able to overcome interference or because in each cell the barrier to infection resulting from interference is not completely efficient. The latter hypothesis would imply that there is a small fraction (four per cent) of the cell surface where the interfering changes do not take place, or that the interfering particle can be dislodged from its critical site by a superinfecting particle with a small probability.

In the remainder of the cells, exclusion cannot be overcome with the highest available multiplicity of the superinfecting virus, i.e. 500.

It would be of interest to determine whether an increase in the multiplicity of the UVI particles would decrease the probability with which exclusion can be overcome in each cell. A positive result would discriminate against the presence in the viral population of particles capable of overcoming interference and would favor the hypothesis that exclusion results from spreading reactions which can be initiated independently of one another at different loci on the cell surface and leave a small fraction of this surface unmodified.

D. Removal of exclusion by anti-NDV serum

To properly discuss the effect of the antiserum in interference, it must be mentioned that exposure to anti-NDV antibody of cells infected by active virus only, seven minutes or later after the attachment of the latter to the cells, does not affect the production of progeny virus; i.e. the infecting ability of active virus becomes insusceptible to homologous antibodies within seven minutes or less.

On the contrary, the interfering ability of the UVI virus is completely removed by specific antiviral serum up to 28 minutes after the addition of the UVI particles to the cells. The effect of the UVI virus is thus completely reversible in all the cells for 28 minutes.

It appears, therefore, that exposure of NDV to UV irradiation renders this virus unable to penetrate into the host cell, at least for 28 minutes. Whether or not this effect is directly responsible for the lack of infectivity of UVI virus remains to be determined.

Normal serum, RDE and trypsin are unable to destroy

interference. This result supports the hypothesis that the inhibition of exclusion by anti-NDV serum is brought about by the specific action of the antiserum upon the UVI particle. This implies that the UVI particles remain at the cell surface and that exclusion takes place at the cell surface.

When anti-NDV serum is added to the cells 30 minutes or more after the UVI virus, only 50 per cent of the cells will support the growth of new virus. The result shows that either 50 per cent of the cells have an irreversible type of exclusion or that the interfering reaction proceeds to such an extent that it results in the death of these cells. Irreversible interference may arise either because the previously reversible changes occurring at the cell surface become "fixed" through some secondary reaction or because in some cells they give rise to physiological changes that progress with the time. Whatever their nature may be, these changes which are of a more permanent nature, occur almost simultaneously in all the affected cells. There is a rather sharp transition from reversibility to irreversibility within a few minutes.

Another question which arises in this connection is whether the cells in which exclusion cannot be overcome by antiserum can be identified with those in which exclusion cannot be overcome by increasing the multiplicity of the superinfecting virus. This question could be solved by exposing cells to a combined treatment. The cells which have adsorbed UVI virus should be first superinfected at high multiplicities of active virus; for instance 10 to 15 minutes after infection with the UVI virus. Then 20 to 30 minutes later they should be treated with anti-NDV serum; at this time the superinfecting virus

has become serum resistant in the cells in which interference was overcome, and the stage for 50 per cent efficiency of the serum on interference has been reached. If the two fractions of cells are identical, 50 per cent of the cells should become yielders; if these fractions arise independently, 75 per cent of the cells should now be yielders. The identity of these two cell fractions would imply that the cell population is divided into two approximately equal parts which react differently from one another to infection by UVI virus as suggested under D.

Some hypotheses which may account for the surface action responsible for interference will now be presented. From the fact that the ratio of the viral surface to the cellular surface is approximately 1:20,000, exclusion seems most easily described in terms of a spreading change at the surface of the cell. Two general types of virus-cell interaction could bring about this change.

One type of change may be brought about by a fast reaction triggered by the UVI virus coming into contact with certain cellular constituents. The spreading time would be short compared to the average time required for infecting a cell with the UVI virus, even at the highest multiplicity of this virus. In this case, the multiplicity effect noted above may be interpreted as a reflection of the different time required to infect all the cells with the UVI virus and to induce in them interference at different multiplicities. The minimal time interval required would represent the time required for one UVI particle to reach a triggering site. Since, with a multiplicity of 140, interference is induced in .1 minute or less,

the interfering reaction is started either as soon as the UVI particle is irreversibly adsorbed (see Section 1) or very shortly thereafter.

Another type of surface change may be caused by a slow reaction; the maximal time interval during which the cell can be superinfected - six minutes or less - would represent the time required for the reaction induced by one particle to spread over the entire cell surface. The multiplicity effect may now be interpreted as an increase in the number of foci where the spreading changes are initiated.

Under either hypothesis such surface changes must be reversible, in order to account for the finding that neutralization of the UVI virus removes exclusion.

The effect of the homologous antibody in removing interference may be brought about by an actual removal of the adsorbed UVI virus particle. This is conceivable, since there are examples in which antibody detaches an influenza virus particle attached to a cell; this effect is however limited to non-infectious virus and to active virus adsorbed onto cells in which virus reproduction cannot take place (6,24). This hypothesis is also supported by the recent findings of Moore and Diamond (75). These authors found that adsorption of NDV to Ehrlich ascites cells in vitro inhibits the formation of tumors in vivo and that the addition of anti-NDV serum to the washed virus cell complexes eliminates this inhibition.

The removal of the UVI particle from its point of attachment to the cell surface may be understood on the basis of a simple model as follows. Many groups on the viral surface form individual bonds with complementary structures on the cell surface. The binding

energy involved in each individual bond may be rather low; the strong binding force between the two particles results from the additive effect of many of these bonds and depends on the structural complementariness of the surfaces involved in this union. Individual bonds may be broken with a relatively high probability per unit time by thermal agitation; but they normally reform, the two bodies being held in place by the other bonds. If specific viral antibodies are present in the medium, they will compete with the cellular receptors for the viral surface group whenever an individual bond is broken. Since it is likely that homologous antibodies have a greater affinity for the viral surface than the elements of the cell surface, they will be preferentially bound to the virus. Eventually enough bonds can be broken to free the UVI particle from the cell surface.

It is also plausible that the adsorption of a single antibody molecule to a viral group which was previously bonded to a cellular group produces a sufficient distortion of the virus surface in the neighborhood to destroy the complementariness between the viral and the cellular surface groups; this may be sufficient to inhibit the effect of the UVI virus, without actually detaching it from the cell.

It would be possible to test whether the UVI virus is actually detached from the cell surface by homologous antiserum through the use of virus labelled with radioactive tracers.

E. Host cell response

So far, the exclusion is the only detectable effect of the UVI particles on the cells. An outstanding fact is the absence of visible morphological damage in the cells to which the UVI virus alone

was attached.

The reported existence of two different types of response to infection by UVI virus in the cell population may be attributed to the existence of different types of cells in the population; differences among the cells may arise among other things, from differences in their physiological states, for instance from the stage in their life cycle.

To learn whether or not the cells showing an irreversible type of exclusion have been killed, the ability of isolated single cells, previously infected with UVI virus, to multiply should be studied. Such a study should be combined with an investigation of the effect of the multiplicity of the UVI particles upon the growth of the host cells and upon the size of that fraction of the cell population which shows irreversible exclusion.

F. Fate of the superinfecting virus

Although exclusion can be completely overcome when antiserum is added up to 28 minutes after the UVI virus, cells superinfected with active virus 9½ minutes after the addition of the interfering agent and then treated with anti-NDV serum 19½ minutes after the UVI virus infection did not yield any progeny virus. This experiment shows that the fate of the superinfecting virus is very different from that of a virus particle infecting a cell to which no UVI particle has attached. In fact the superinfecting virus either becomes inactivated after its adsorption to the resistant cells or is prevented from penetrating the host cell, thereby remaining susceptible to neutralization by specific antibodies. Again, through the use of virus labelled with radioactive tracers, it should be possible to distinguish

between these two alternatives.

Additional information on this point is given by observations which do not involve the use of antiviral serum. It was found on one hand that there is on the average a 20 fold drop in the amount of free active particles eluted from cells which received UVI virus prior to the active one when compared with similar supernates of normally infected cells. It appears from this result that the superinfecting virus is either destroyed or becomes unable to elute. On the other hand, a cell infected by active virus under conditions in which the latter is excluded, does not yield active virus during an incubation period lasting for six days. Since exclusion eventually disappears 20 to 60 hours after the infection of the cell with UVI virus, the superinfecting virus must become in some way inactivated. Thus all the available evidence concurs in showing that in this form of interference the excluded virus becomes inactivated perhaps on the surface of the cell.

FIGURES

1. Rate of adsorption of B-NDV to chicken embryo cells at $37^{\circ}C$ at pH 7.4.
2. Survival curve of B-NDV irradiated with UV light.
3. Growth curves of B-NDV in NMI. The time given starts at the moment the yielders were resuspended in NMI.

   Curve A: Control (active virus only)

   Curve B: UVI virus prior to active one

   Curve C: UVI virus only
4. Growth curves of B-NDV in NMI at various concentrations of the interfering agent. The time given starts at the moment the cells were superinfected with active virus.

   Curve A: Control (no UVI virus)

   Curve B: 5×10^{7} UVI particles prior to active virus.

   Curve C: 1×10^{8} "

   Curve D: 2×10^{8} "
5. Determination of the multiplicities of infection as a function of the input concentration of active virus. The ordinate is the fraction of cells which did not yield any progeny virus.
6. Determination of the number of particles required to induce exclusion.

   Curve A: Theoretical plot ofequation 3 section V

   Curve B: Theoretical plot of equation 4 section V.

   The experimental points represent the fraction of cells which became yielders when superinfected with active virus as a function of the multiplicity of the UVI virus.

7. Determination of τ . The straight lines represent the theoretical curves of $(1-\frac{C_\infty}{C_o})$ versus V_a for different values of τ according to equation 12 section VI-B.
Figure 7 A is valid for $V_i = 2 \times 10^9$ UVI particles per ml. and Figure 7 B for $V_i = 6 \times 10^9$ UVI particles per ml.

8. Effect of adding UVI virus after the active one. The time given starts at the moment the cells were infected with active virus.

   Curve A: Control (no UVI)

   Curve B: UVI virus added 72 minutes after active virus.

   Curve C: UVI virus only.

9. Duration of exclusion. The time given starts at the moment the cells were infected with active virus.

   Curve A: Control (no UVI)

   Curve B: UVI virus 13 hours prior to active virus

   Curve C: UVI virus only.

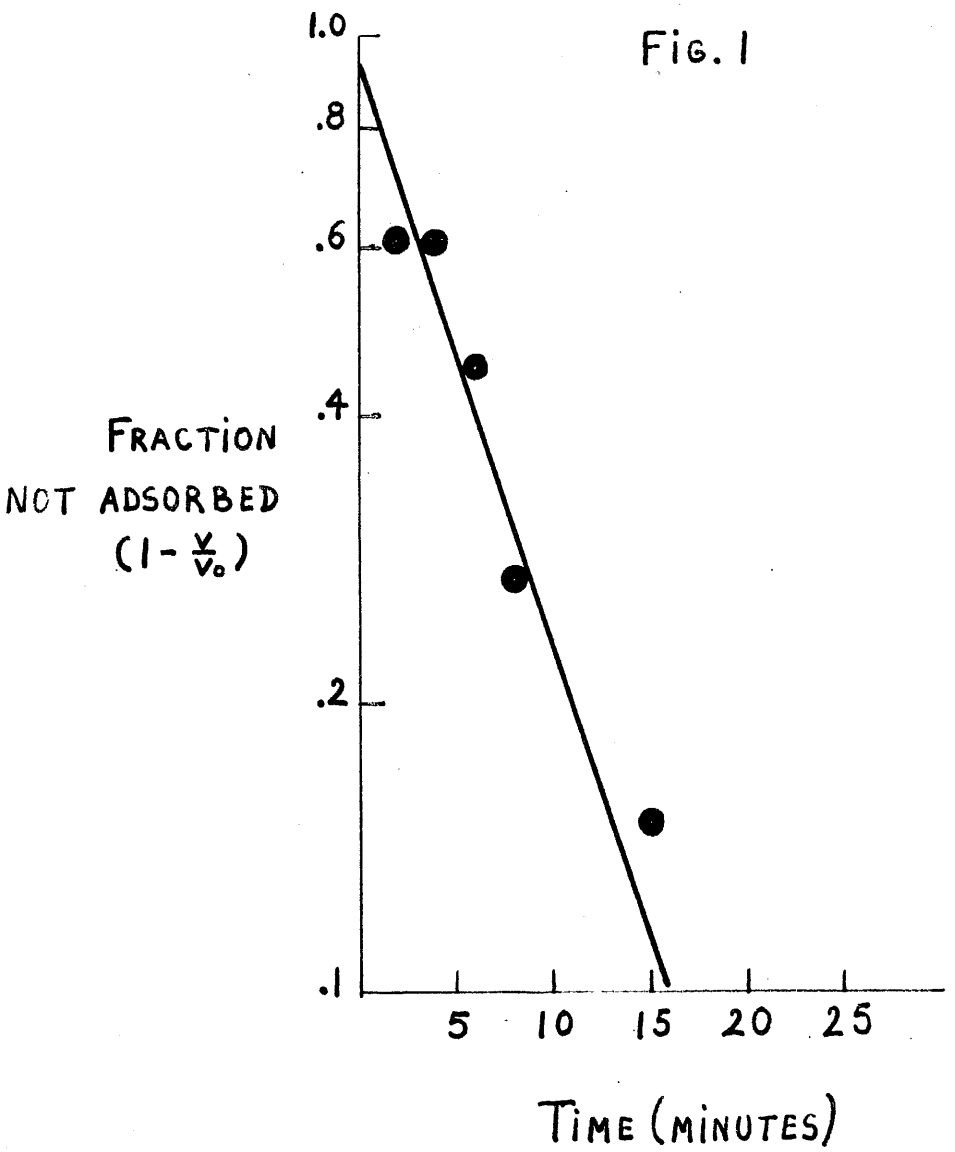

1.0
.8
.6
.4
.2
.1
FRACTION
NOT ADSORBED
$(1-\frac{V}{V_0})$
5
10
15
20
25
TIME (MINUTES)

FIG. 1

Fig. 2

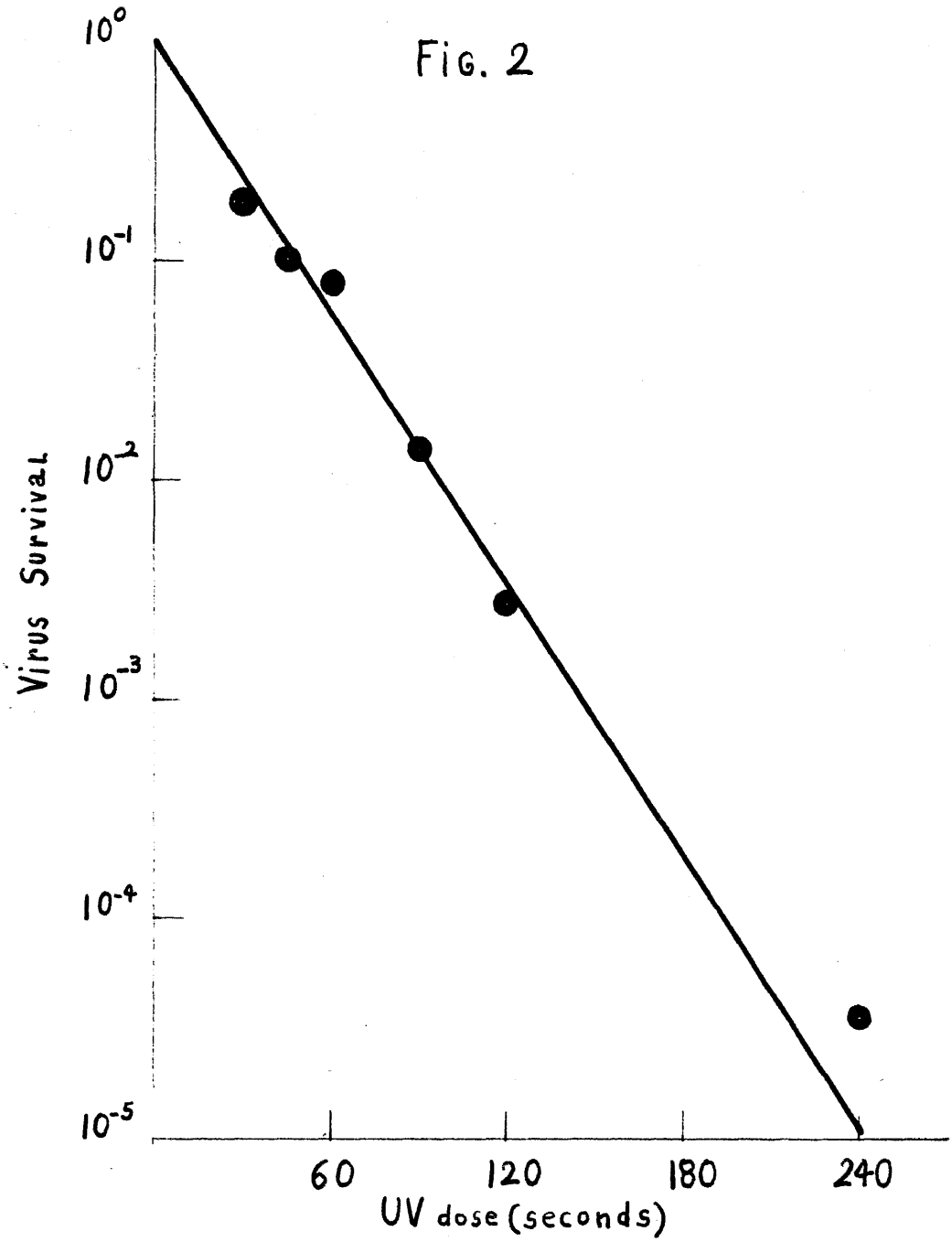

10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
Virus Survival
60
120
180
240
UV dose (seconds)

Fig. 3

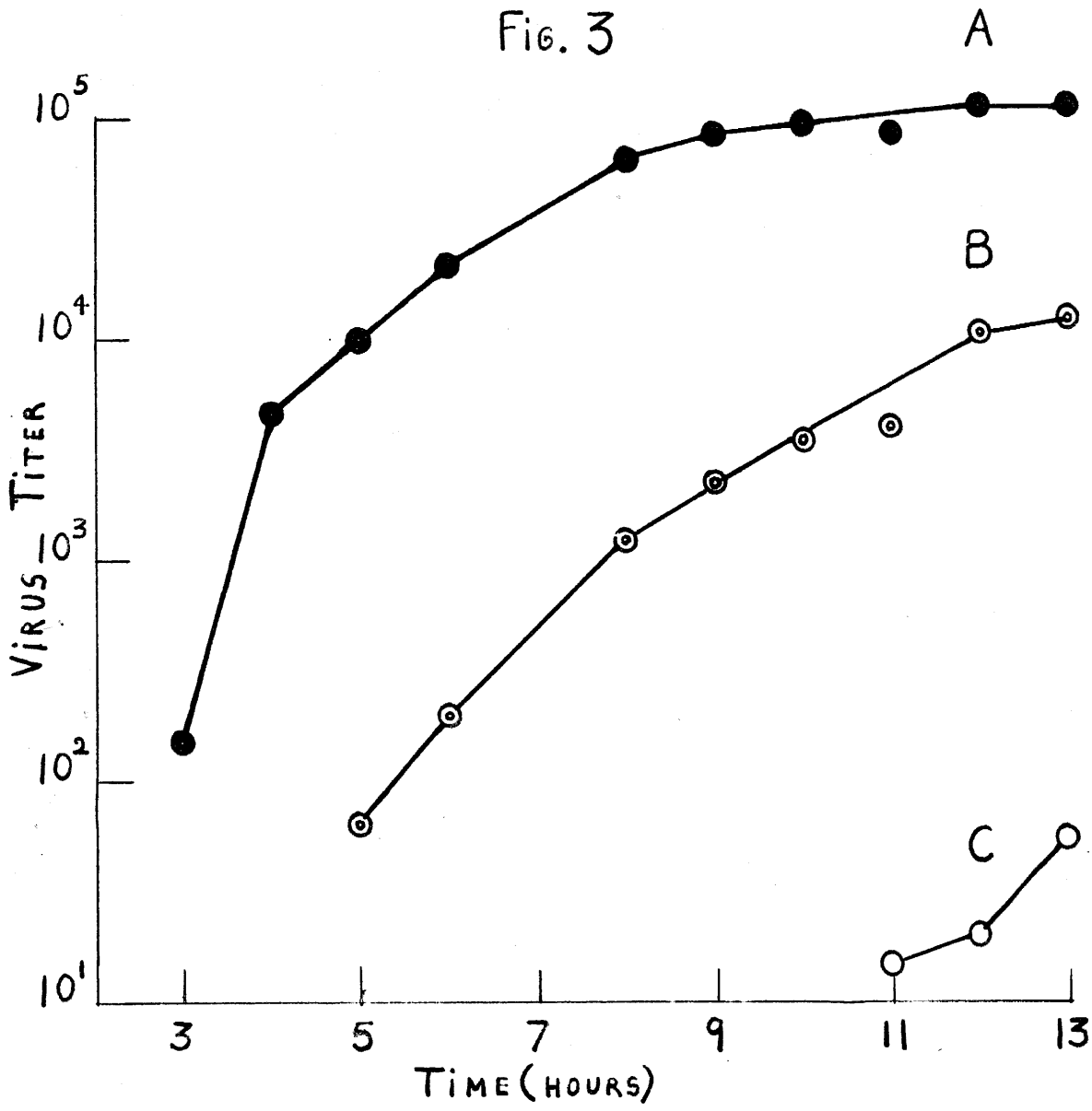


Fig. 4

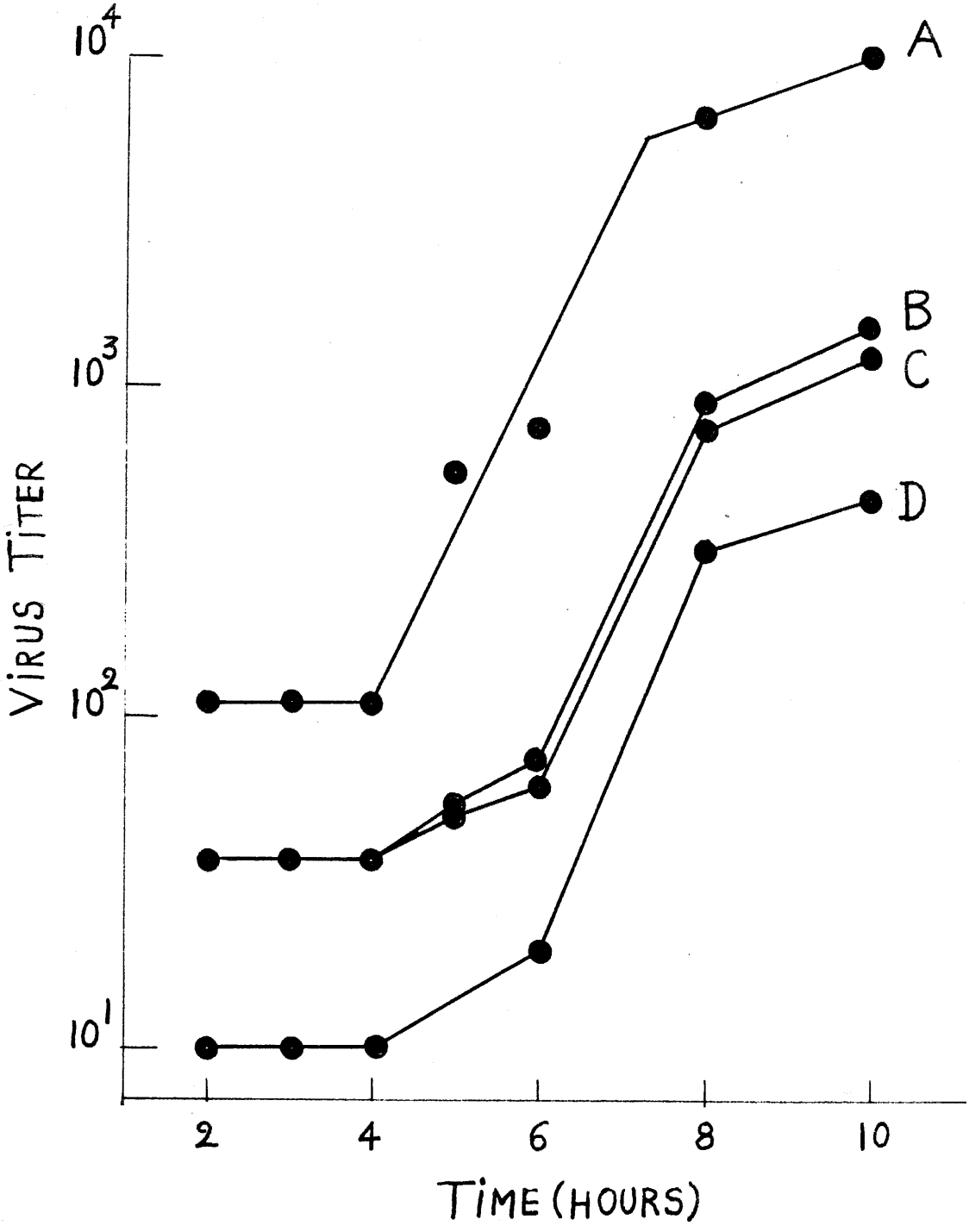


Fig. 5

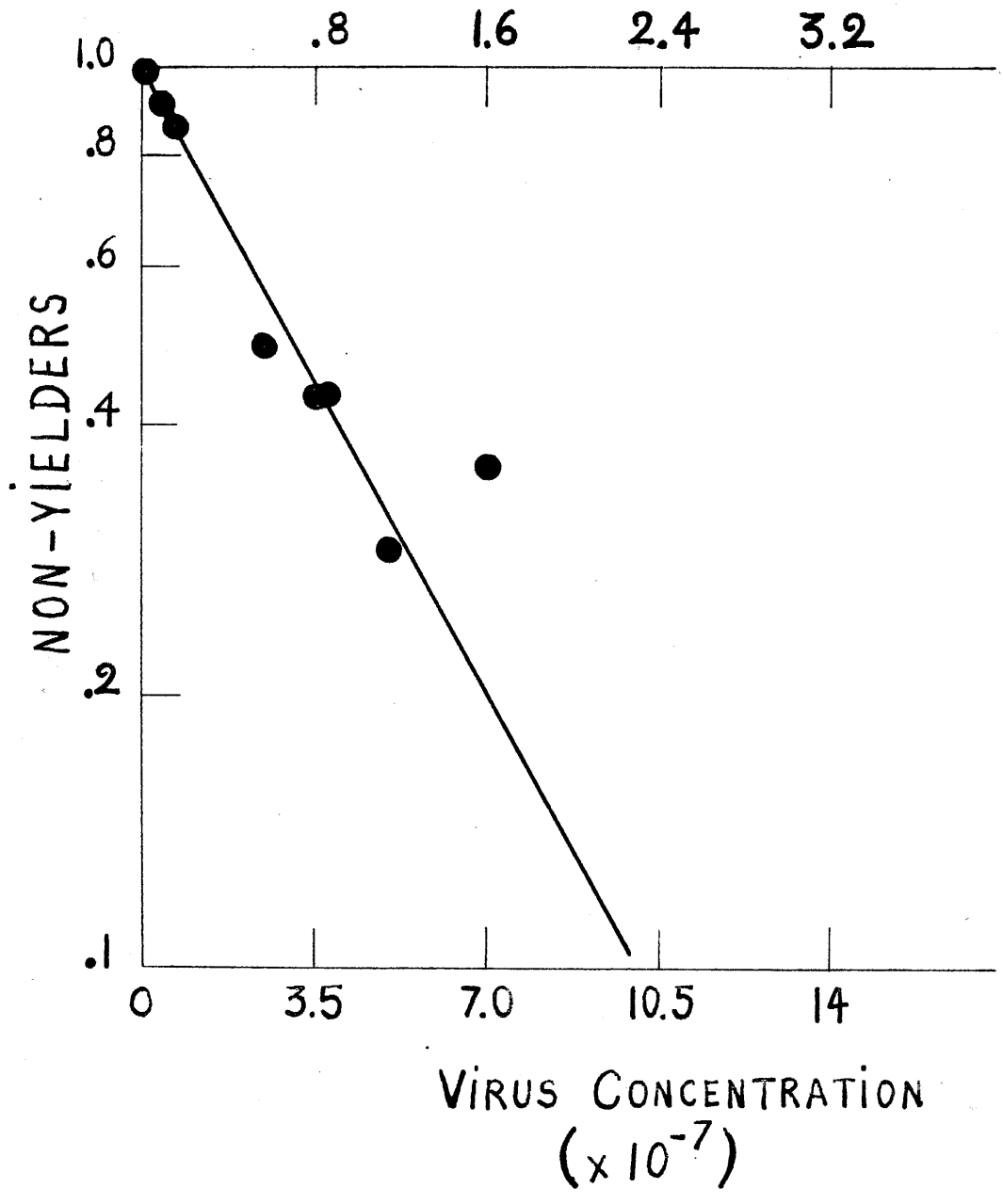
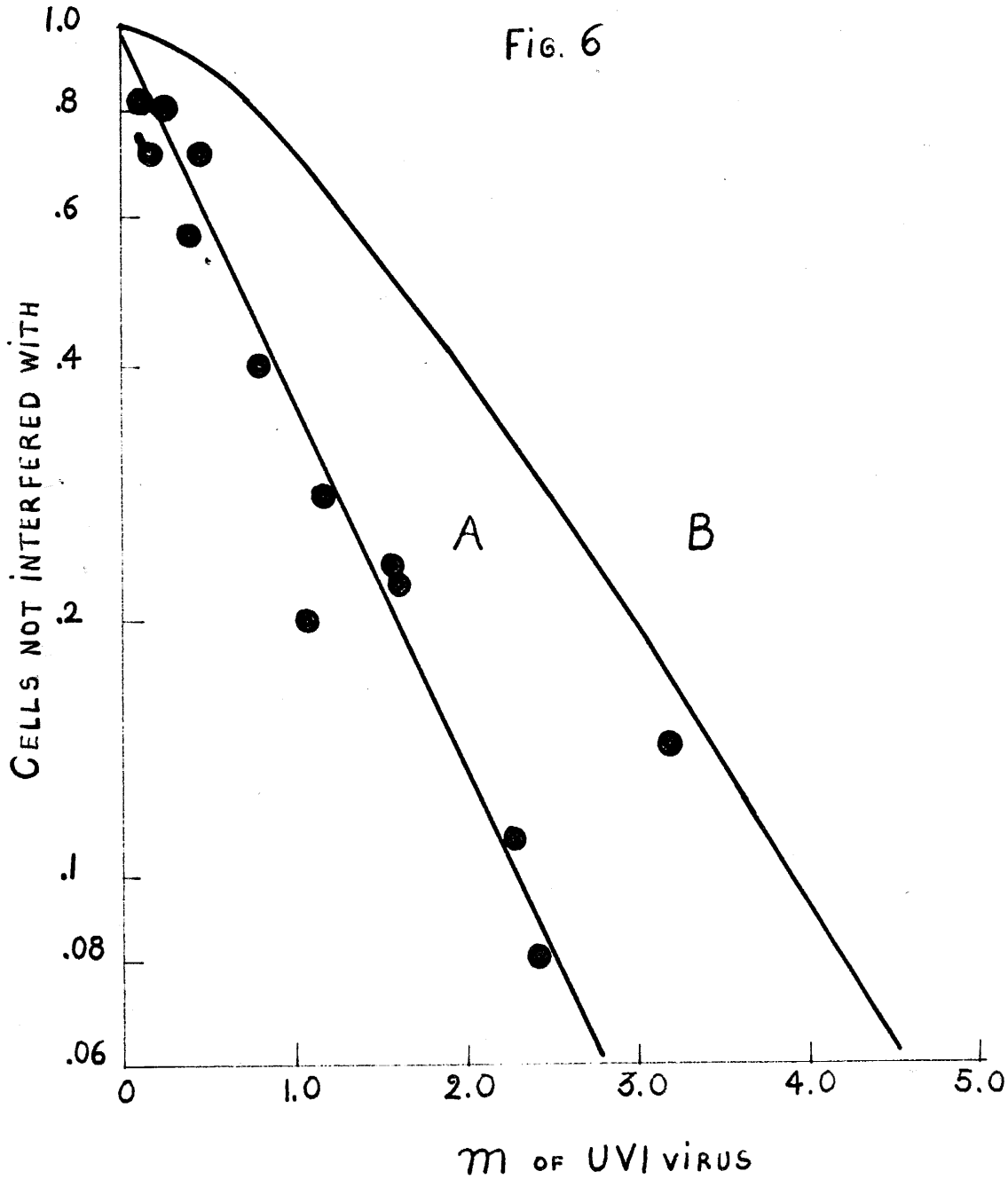

m
.8
1.6
2.4
3.2
1.0
.8
.6
.4
.2
.1
NON-YIELDERS
0
3.5
7.0
10.5
14
VIRUS CONCENTRATION
(x 10⁻⁷)

Fig. 6


1.0
.8
.6
.4
.2
.1
.08
.06
CELLS NOT INTERFERED WITH
A
B
0
1.0
2.0
3.0
4.0
5.0
m OF UVI VIRUS

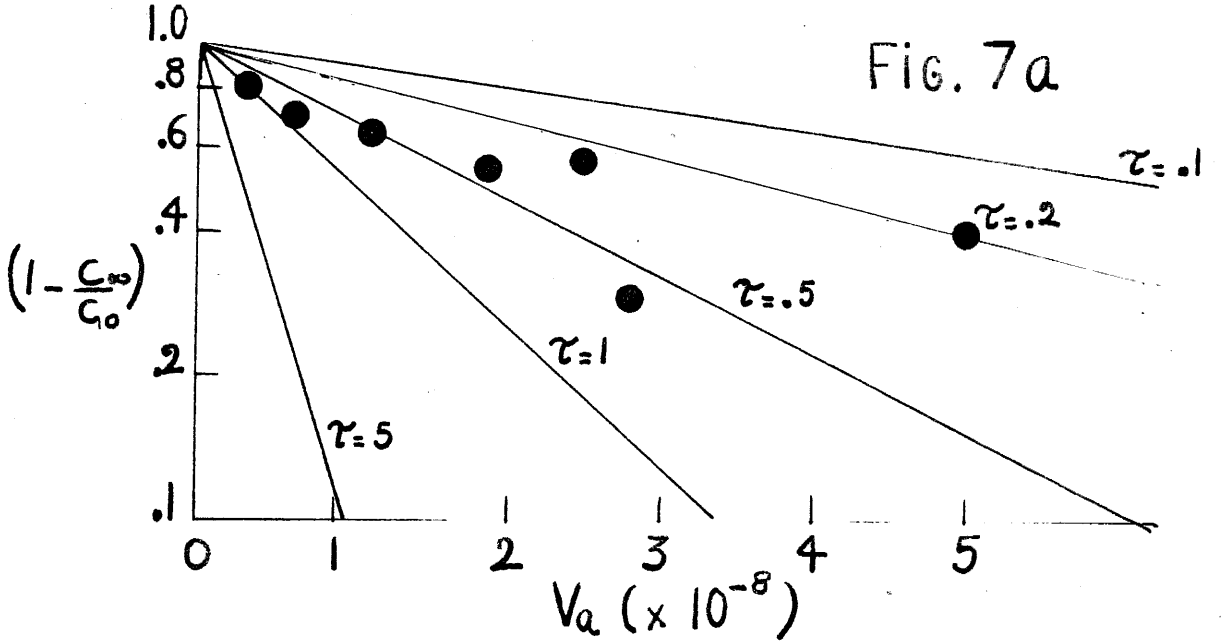

1.0
.8
.6
.4
.2
.1
$\left(1-\frac{C_\infty}{C_0}\right)$
τ=.1
τ=.2
τ=.5
τ=1
τ=5
0
1
2
3
4
5
$V_a\ (\times 10^{-8})$

Fig. 7a

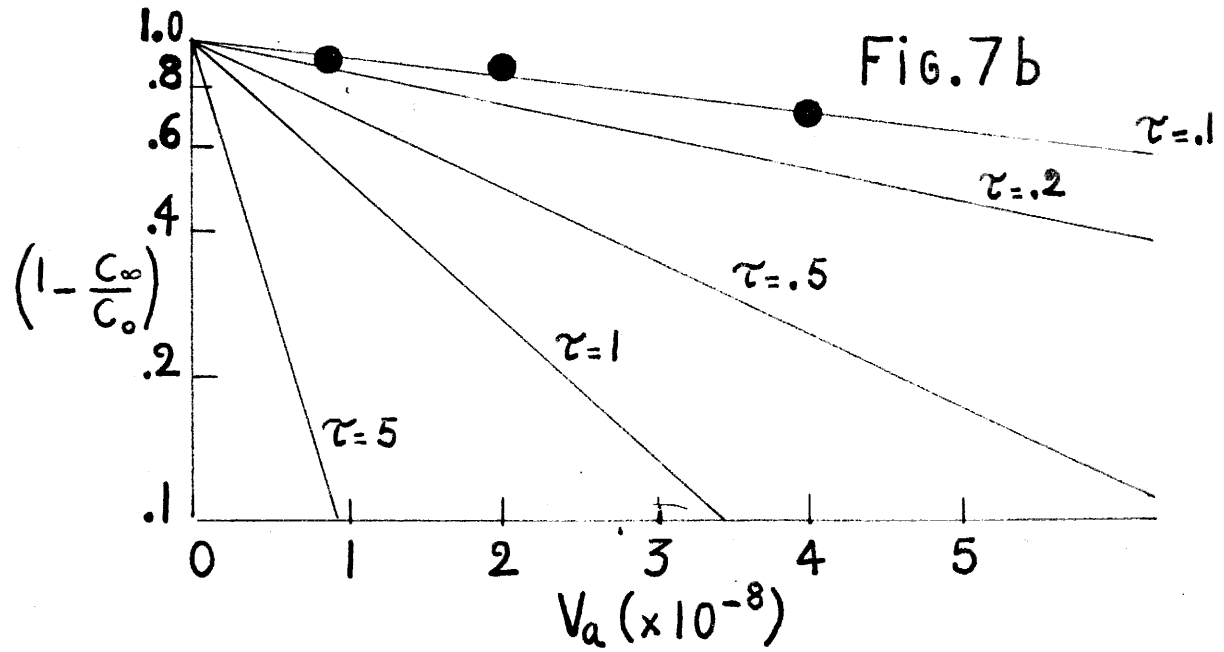

1.0
.8
.6
.4
.2
.1
$\left(1-\frac{C_\infty}{C_0}\right)$
τ=.1
τ=.2
τ=.5
τ=1
τ=5
0
1
2
3
4
5
$V_a\ (\times 10^{-8})$

Fig. 7b

Fig. 8

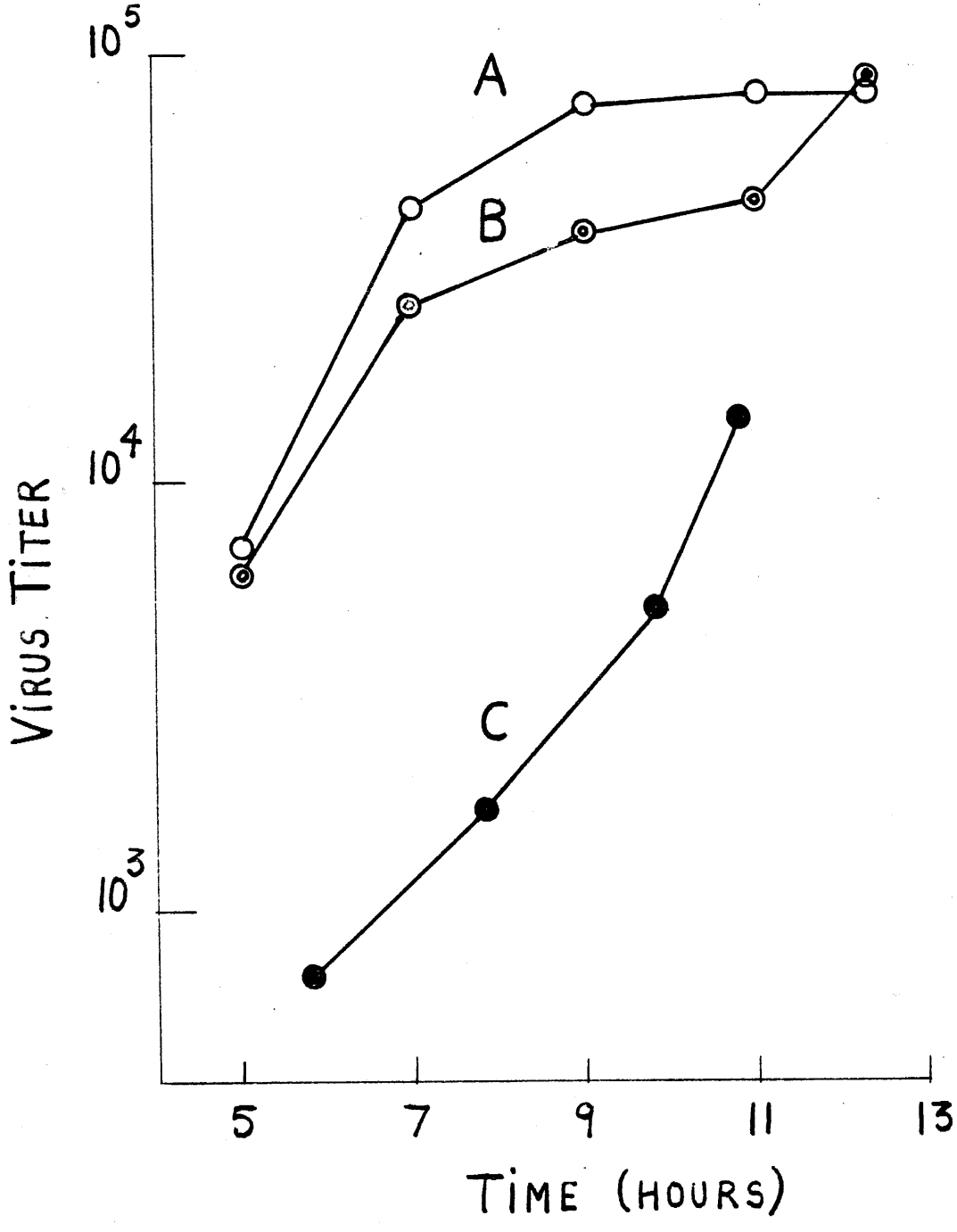
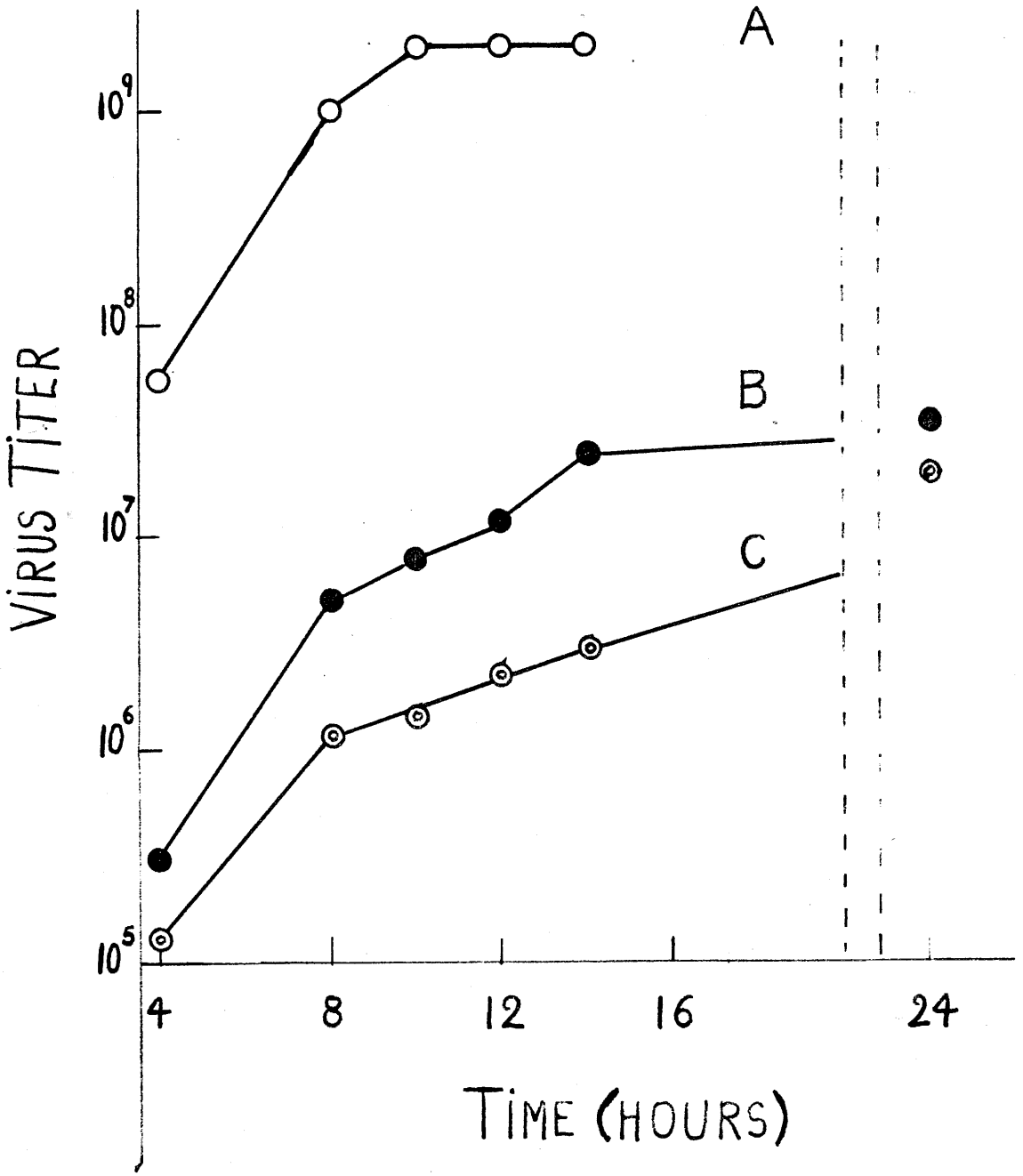


FIG. 9



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