AN INVESTIGATION INTO THE ABILITY OF INTRACEREBRALLY INOCULATED MYXOVIRUSES TO PROTECT AGAINST INTRAPERITONEALLY ADMINISTERED ARBOVIRUSES AND THE DEVELOPMENT OF AN HYPOTHESIS CONCERNING INTERFERON INDUCTION

by

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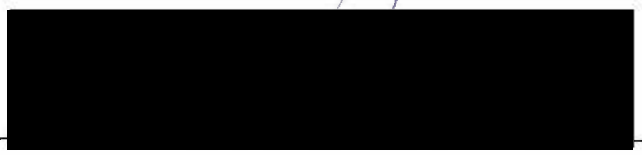
Approved: 

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AN ABSTRACT OF THE THESIS OF

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A major drawback to the use of arboviruses as oncolytic agents is their often lethal neurotropism. A previously noted protective phenomenon induced by intracerebrally inoculated myxoviruses could possibly selectively protect the brain from the neurotropic effects of oncolytic arboviruses. To investigate the phenomenon, a number of myxoviruses and arboviruses were used. A model system using Newcastle Disease virus (NDV) and Sindbis virus in white Swiss mice was finally established with which to explore both the extent of the phenomenon and its mechanism of action.

It was shown that a protective index of over $10^{6.4}$ was possible against an intraperitoneal challenge by Sindbis. Significant protection was established within eight hours and persisted through ten days. The magnitude of protection was directly related with the number of interferon units induced in the brain, at least when the protective index was $10^{3.1}$ or less. Significant protection also resulted from ultraviolet

(UV) irradiated aliquots of NDV containing a low infective titer of virus.

Comparison of irradiated and diluted NDV samples of matched infectivity revealed that neither infectious virus, nor particles rendered noninfectious through UV irradiation were responsible for the induction of interferon in the mouse brain. UV-inactivationcurves also indicated that the NDV was comprized of two or more virus populations with differing UV sensitivity.

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Introduction

It has been known for many years that viruses will grow readily in a number of transplantable animal tumors. In some instances the effects of this infection has been varying degrees of inhibition of tumor growth. For example, in 1923 Levaditi and Nicolau reported that a neurotropic strain of vaccinia would interfere with the growth of several types of animal tumors (1). To date over ninety such virus-tumor relationships have been studied (2, 3, 4).

In 1950, Koprowski and Norton (5) screened many viruses to determine which of these possess oncolytic properties and for which tumors. Mice bearing various transplantable tumors were challenged with subcutaneous (sc) or intraperitoneal (ip) injections of one of fourteen neurotropic viruses. The viruses of Louping Ill, Ilheus, West Nile (WN), St. Louis encephalitis and Russian Spring-Summer encephalitis (RSSE) parasitized the transplantable mouse sarcoma 180 (S180) and showed partial or total oncolysis of tumor cells, as judged by vital staining and transplantability. The viruses of Semliki Forest, Bunyamwera and Japanese B encephalitis showed no oncolysis despite the presence of the viruses in tumor tissues. Eastern equine encephalitis was the only one of a group of fourteen other viruses which caused encephalitis without parasitizing the tumor tissue. No effect on S180 was observed with western equine encephalities (WEE), pseudorabies, French neurotropic yellow fever, Passos I, Ntaya and Bwamba viruses which failed to infect the mice by parenteral inoculations.

A limited number of experiments using the MCI fibrosarcoma and the EO771 (mammary) adenocarcinoma gave results similar to those found with S180.

Against two osteogenic sarcomas (Wagner and Ridgeway), the most consistant inhibitory effect was observed with WN and Venezuelan equine encephalitis, and in one experiment, with Bunyamwera virus.

Moore (6, 7) similarly tested the effects of the Group B arbovirus, RSSE, against several transplantable mouse tumors and extensively studied the oncolytic relationship which exists between RSSE and S180 (8, 9, 10).

Viral oncolysis has likewise been attempted in humans bearing heterotransplantable and primary neoplasms (11, 12). In general, viruses used in humans have either been those which are nonpathogenic for man, ie Newcastle Disease virus (NDV), bovine enterovirus; those commonly acquired and/or causing a mild disease, ie vaccinia, varicella, mumps; or occassionally more pathogenic organisms of man against which the recipient had been immunized, ie measles and poliovirus.

Important findings have been established by the preceding studies and may be summarized.

1) In order to produce an oncolytic effect, a virus must actually parasitize and replicate in the tumor itself. The susceptiblity of the tumor to viral infection is subject to the same criteria of humoral immunity to which the host is subject.

2) In general, no obvious relationship between tumor and virus, in an oncolytic pair, has been established and the liklihood of oncolysis of any particular

tumor by a particular virus can not be predicted (5).

3) With respect to transplantable mouse tumors, several arboviruses are capable of oncolysis. Again, however, no obvious relationship between tumor and virus has been established (Table 1).

For certain tumors, an encephalitis virus may be more "oncotropic" than neurotropic, in that the titer of virus in the tumor reaches a maximum earlier than in the brain, although the same titer is finally achieved in both (Table 2) (8). This results in destruction of the tumor up to three days before destruction of the brain, depending upon the dosage of virus administered (Table 3). While this situation presents many facets for further investigation, a problem of immediate practicality is illustrated by Moore's work (6-9, 13). The problem was stated by her in 1950 (10).

> Although the tumor may be destroyed some days before the death of the animal, the virus eventually attacks the brain, producing paralysis and death. Attempts to separate the lethal and tumor-destroying ability of the virus have so far been unsuccessful..... It is.....useful to know that the age of the tumor and route of inoculation make no difference in the ability of the virus to cause tumor destruction.

It is distressing that there exists an effective method of oncolysis (Table 3), a principle which might be greatly expanded were it not for the persistant lethal neurotropism. Attempts to develop an attenuated RSSE strain with decreased neurotropism have been unsuccessful (9). However, in 1947, Vilches and Hirst (14), while investigating the phenomenon of viral interference, noted that an influenza virus injected ic could protect against an encephalitis virus injected by the same route. Although the mechanism of interference was unknown, they investigated the onset, duration

and extent of protection, using several species and strains of viruses.

My own treatise describes an investigation into the mechanism of viral interference of the type noted by Vilches and Hirst, and an exploration into some of its potentialities. Because of the possibility of ultimately exploiting this mechanism to achieve a safe oncolysis with neurotropic viruses, some attempt to determine the relationships among a transplantable mouse tumor, various arboviruses, and myxoviruses was made.

The interference noted by Vilches and Hirst has several peculiar characteristics. They found that protection against more than 10^5 fifty percent lethal doses (LD_{50}) of the Lederle strain of WEE virus is established within three hours and lasts at least seven days when the PR8 strain of influenza is used. Other viruses, including inactivated PR8 virus, were also able to provide some protection. The variability in the degree of protection was unexplained.

The rapidity with which the protection is established, the non-specificity of the interference, and its induction by inactivated virus suggests that this protection is mediated by an interferon (15). However, the magnitude and duration of the protection (protective index greater than 100,000 for seven days) are not generally noted characteristics of an interferon mediated protection (16-20).

Interferon is a species-specific protein produced by cells after their exposure to active or inactive virus or double-stranded ribonucleic acid (RNA) which renders cells resistant to virus infection (21-33). The exact mode of

action of interferon is still a matter of some conjecture but is known that interferon brings about an inhibition of RNA-synthetase involved in synthesis of viral but not normal cellular RNA.

The mechanism of interferon induction in cells is likewise incompletely understood, although for interferon production to occur some double-stranded RNA must enter the cell. How interferon induces antiviral activity in cells not exposed to foreign RNA is unclear, although it has been hypothesized to exert its protective action through a second mediator, "translation-inhibitory protein" (TIP) (34, 35).

It is generally agreed that interferon production in in vivo infections ceases by the 36th hour and the level of circulating detectable interferon falls off rapidly about six hours after intravenous or intraperitoneal inoculation (15, 36, 37, 38). What limits or inhibits further interferon production is unknown. The persistence of protection well past seven days as noted by Vilches and Hirst contrasts with the usually noted absence of detectable interferon beyond 36 hours. It is also unusual for interferon to provide greater than 100-fold protection in vivo (39, 40).

Vilches and Hirst further noted a marked variability in the protection afforded by different viruses and by the same virus against different challenging agents. This variability must be understood for standardization of protection.

If a safe method of viral oncolysis by neurotropic viruses through this mechanism is to be achieved, answers to the following are prerequisite. Is a particular type or form of virus necessary? Are the infectivity, viability, pathogenicity

or titer of the virus important factors for the protection? Is protection mediated by or related to interferon? Is the protection a phenomenon which is local, humoral or uniformly distributed throughout host tissues? Since, in oncolytic therapy, challenge virus would need to be administered via a route other than intracerebral (ic), is protection effective if the challenge is given via other routes? With these questions in mind, it is pertinent to review some of the present knowledge of central nervous system (CNS) infections, and ic injections.

In this discussion the term neurotropism refers to the neurologic component of systemic viral infections. It does not imply a special affinity for neural tissues but does specify the ability to produce symptomatic neural disease.

Naturally acquired neurotropic virus infections and virus inoculated experimentally by routes other than ic must gain entry to the CNS by some natural pathway. It has been shown that at least three general entryways exist and that various viruses may use any one or even all three, depending upon the particular virus-host relationship (41).

Viruses may gain access to the CNS via peripheral nerves using axons, perineural and endoneural cells, lymphatics, or tissue spaces. A second pathway is via the olfactory mucosa using mucosal and submucosal cells to spread to the subarachnoid cuff surrounding olfactory neurons and thus through the cribiform plate to the meninges, or using endoneural and perineural cells of the olfactory fibers to cross the cribiform plate to the parenchymal cells of the olfactory bulbs, or as has been suggested by studies of aerosol infection

such as those with West Nile virus (42), by spread along olfactory fibers without apparent infection of submucosal, endoneural or perineural cells.

The third method of entry is hematogenous. It is the prime route of arboviruses infecting the CNS. Their entry first requires a viremia of sufficient magnitude and duration. For maintainence of this viremia, virus must be shed into the blood from a multiplication site in some extraneural tissue. The attainment of a sufficient viremia allows penetration of what has been termed the "blood-brain barrier" (43).

Although a variety of mechanisms have been postulated to explain how viruses cross from blood to brain, it should be noted that theoretically no active mechanism is required, for Shultz and Frohlich (44) have demonstrated small amounts of bacteriophage in the cerebrospinal fluid (CSF) of dogs following large intravenous injections, and micromolecules of ferritin have been shown to cross readily from the CSF into the parenchyma of the brain (45). However, both Japanese encephalitis virus and neuro-adapted strains of influenza administered extra-neurally infect cells of the mouse choroid plexus, thus allowing infection to spread via the CSF to the ependyma and thence to the brain parenchyma (41, 46).

The small capillary blood-brain junction is the site which is termed the "barrier." Even so, macromolecules of ferritin can pass readily from blood into the brain through this junction (47). Although in theory neurotropic arboviruses could gain entry to the CNS in such a fashion, West Nile and Sindbis viruses have been demonstrated to infect their way from the blood stream via the vascular endothelium to the

surrounding neurons and glial cells (41, 48).

Within the central nervous system itself there is a heterogeneity in the susceptibility to infection. Each neurotropic virus has a spectrum of cells which it can infect. These may often be characterized by their different anatomical location or function (Table 4). However, some viruses are even more selective, as demonstrated by the specificity of polio-myelitis virus for motor neurons, arboviruses for cells of gray matter, yellow fever virus for mouse astrocytes or fixed rabies virus for Purkinje cells of the mouse cerebellum.

For a cell to be susceptible to any virus it must be capable of specifically absorbing the virus to its cell membrane, transporting it across the membrane and releasing the viral nucleic acid, in addition to replicating the virus components. The presence or absence of specific receptor sites for the absorption of a particular virus to the cell membrane is apparently the most important factor in susceptibility (48-52). That these cell-surface receptors are crucial in determining not only susceptibility of different tissues and hosts, but also of the same host or tissue at different ages is indicated by the fact that the decrease in receptor site activity for Sindbis virus in mouse brain has been shown to parallel the increasing resistance to disease in vivo (41). Similar findings have been reported for changes in enterovirus susceptibility of primate tissues with aging, culturing or disrupting normal surface to surface cell contacts (53-56). The preponderance of evidence supports the concept that animal viruses attach to specific membrane receptors which determine cellular susceptibility and are then transferred into the cell

by an active but nonspecific mechanism.

A number of things are known about viruses inoculated intracerebrally. Any material inoculated into mice by this route flushes the spaces available to the CSF by backwashing along the injection tract (41). No pocket is formed within the brain parenchyma. The inoculum quickly becomes dispersed throughout spaces available to the CSF (including the perivascular spaces), bathing the ependyma and meninges and, as the hydrostatic pressure of the CSF rises, passes into the venous bloodstream by rupturing through the arachnoid villi. When any virus capable of growth in ependymal or meningeal cells is inoculated ic, widespread infection of meninges and/or ependyma throughout the entire CNS always precedes infection of parenchymal cells. In the case of non-neuroadapted influenza viruses, no parenchymal infection occurs, although a coating of infected cells completely surrounding the parenchyma of the brain is established, usually asymptomatically.

A "toxicity" effect has been reported upon ic injection of large doses (10^8 50% infective doses) of some standard strains of influenza virus (46) which can cause death in mice, usually within seven days. This toxic effect may also result from NDV, however the toxicity can be prevented by prior injection of receptor destroying enzyme (RDE) which has the action of removing specific receptor sites for influenza (and NDV) viruses from cell membranes. In mice so injected there is a great reduction in the percentage of ependymal and arachnoid cells which produce viral antigen following ic inoculation of influenza.

In spite of an overflow of virus into the systemic circulation

during ic injections, the titer of interferon in the brain has been recorded up to 1000-fold greater than in the blood or non-neural tissues (38). Thus, it would appear that ic injection of non-neurotropic virus should be a rather effective method of selectively protecting the brain against a neurotropic virus by any route. Surprizingly, when this was tried (4), using NDV as a protecting agent against three arboviruses, only small multiples of the LD_{50} of the arboviruses were able to be given without an ensuing encephalitis, the greatest protection being recorded as roughly a 100-fold difference between the NDV-inoculated mice and controls. Usual protection was less than 10-fold. The variability in the effectiveness of protection was unexplained.

This cited study was an attempt to protect the brain against arboviruses capable of oncolysis of the Erlich ascites carcinoma in mice. For the most part no absolute protection could be demonstrated in animals with tumor cells which became infected with arbovirus. It is nearly certain that infection of the tumor cells produced a marked viremia, much greater than that produced by low doses (30 LD_{50}) of virus in non-tumor bearing mice. This viremia, of sufficient high titer and duration, allowed infection of the CNS. If a virus inoculated ic is to protect the CNS against the onslaught of a marked and persistant arbovirus viremia maintained by infected tumor cells, a protection of the type noted by Vilches and Hirst is required.

The subject of this research is to explore the method of protection noted above for it's possible use in viral oncolysis.

MATERIALS AND METHODS

Materials

Viruses: All viruses used are available from either the Arbovirus Reference Laboratory or the Respiratory Virus Infections Unit, National Communicable Disease Center (NCDC), United States Public Health Service, Atlanta, Georgia. Arboviruses, except as noted, were passed upon receipt by ic inoculation of a 10^{-2} dilution in newborn mice. Arbovirus preparations were lightly centrifuged 20 per cent mouse brain suspensions in five per cent bovine albumin in phosphate-buffered saline (BAPBS) at pH 7.8-8.0. The technique was as described in the NCDC laboratory manual[1] with the exception that brains from frozen and thawed carcasses of mice sacrificed when moribund, were harvested by aseptic aspiration. Virus was stored in sealed glass ampules at -70° C. For mouse inoculations, appropriate 10-fold dilutions of the 20 per cent suspension were made in 0.75 per cent BAPBS containing, at a final concentration per ml, 200 U of penicillin G potassium (Pfizer) and 100 ug of streptomycin sulfate (Pfizer).

Unless noted, myxoviruses were passed upon receipt by chorio-allantoic inoculation of a 10^{-2} dilution in nine-day-old embryonated hen's eggs. Diluent for inoculations was Hanks solution containing 200 U of penicillin G potassium

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[1] Laboratory Techniques in Virology, NCDC, Atlanta, Georgia, 30333.

(Pfizer) and 100 ug of streptomycin sulfate (Pfizer). Infected eggs were incubated at 35° C for two days, refrigerated for 14-18 hours and harvested, pooling the amniotic and allantoic fluid from each egg. Hemagglutination (HA) titrations were done with 0.5 per cent triple-washed chicken erythrocytes in phosphate-buffered saline (PBS) at pH 7.4.

Arboviruses: The passage history of these viruses is designated as the number of passage (P). When the passage host is known, the number of passages in that host, usually suckling mouse (SM), is given.

WEE, Fleming strain, not passed upon receipt, SM6.
WEE, NJo-138 D strain, not passed upon receipt, SM3.
WEE, Cox strain, passage history unknown, believed to have been originally obtained from Lederle Laboratories.
WEE, 558 strain not passed upon receipt, P11.
Sindbis, AR1055 strain, SM11
Guaroa, SM2
West Nile, AR248, SM3
Langat, TP-21 strain, P2 SM2
Ilheus, P26, SM1
Louping Ill, SM1

Myxoviruses: The passage history of each of these viruses (Table 5) is designated by the number of passages in each host, such as ferret (F), mouse (M), Monkey kidney (MK), or embryonated hen's eggs (#).

Influenza A/PR8/34, F8/M593?E170.
Influenza A/Swine/1476/31, M38/E48.
Influenza B/Lee/40, F8/M13?/E186.
Influenza B/MD/59, MK2/E1/MK1/E11-14.
Influenza B/Den/59, E4-7.
Newcastle Disease virus (NDV)/NJ Roakin, E20-23.

Nucleic Acid: A synthetic double-stranded ribonucleic acid (RNA) polymer of inositic and cytidylic acids prepared by P-L Biochem[2] was obtained from the Developmental Virology

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[2] P-L Biochem Inc., 1037 W. McKinley Ave., Mil., Wis., 53205

Unit, NCDC. The manufacturer specifies 20 per cent by weight is in the biologically active double-stranded form. An 80 mg % RNA solution in 80 mg % DEAE (di-ethyl-amino-ethylene dextran) MW approx. 2 x 10^6) in PBS, designated P:IC-dx (Table 5), was used in one experiment following Dianzini's example.(57).

Methods

Titrations: Unless otherwise noted, all arbovirus titrations were done in suckling white Swiss mice. Titers are expressed as 50 per cent lethal doses (LD_{50}'s) and endpoints and their standard deviation (SD) were determined by the Spearman-Karber method (58). Myxovirus titers were determined as 50 per cent egg infective doses (EID_{50}'s). Evidence for egg infection was the presence of 4+ hemagglutination in a 1:2 dilution of the harvested allantoic and amniotic fluids in PBS. HA titers were also determined where harvested fluids were to be used for other purposes.

Inactivation: Ultraviolet (UV) irradiation of virus was done by placing about 2.5 cc of freshly thawed liquid preparation (or an amount sufficient to cover the bottom) in 100 x 15 mm Petri dishes. These were placed on an agitator under a biological safety hood at a distance of 24 cm from a 15 watt G. E. germicidal lamp. UV exposure was timed by a stop watch. After UV irradiation, a portion of each preparation was diluted and inoculated into eggs for EID_{50} titration and the balance frozen and stored until use.

Experimental Design: Except where noted, the following procedure was used: suckling litters were redistributed so

that each litter in a test group contained no siblings but sucklings in each litter had a sibling in every other litter of the group. Most commonly the litter size was eight but in some experiments other litter sizes varying from 6 to 10 were used. The agent to be tested for interference was injected ic in a volume of 0.02-0.03 ml. Twenty-four hours later, challenge virus was titered in litters of both test and control groups by giving 0.05 ml ip of one of the serial dilutions of the challenge arbovirus. Mice were observed daily and any deaths recorded each day, excepting any during the first 24 hours after challenge. These were not included in calculations. Experiments were terminated on the fourteenth day.

Controls using normal chick embryo allantoic and amniotic fluids, Hanks solution, and 0.75 per cent BAPBS as protective agents showed no protective effect.

Interferon assay: Assay of the biological units of interferon in mouse brains following the ic inoculation of the protective agent was done through the courtesy of Dr. E. R. Borden, Developmental Viology Unit, NCDC. Preparation of material for assay was done by homogenization of frozen and thawed, aseptically aspirated mouse brains in Hanks solution to make a 1:8 dilution. This was lightly centrifuged, decanted, and the supernatant frozen until submission for assay. Assay of biological activity was in L cells using Mengovirus. Interferon was reported as units, with one unit being the amount of interferon necessary to decrease a plaque count by 50 per cent.

EXPERIMENTAL AND RESULTS

In the first group of experiments, Influenza B/Den/59 was tested against a series of seven arboviruses. This myxovirus was chosen because of previous experience with it against a strain of WEE in which protection up to 10^5 LD_{50} had been achieved. The results of this group are summarized in Table 6. Here, and in some following experiments, the endpoint in either the experimental or control titration was missed, allowing only a minimal statement as to degree of protection. However, even with a relatively low infective titer of myxovirus, a significant degree of protection is afforded against all challenge viruses. In no case is the difference between the arbovirus titers in the test group and the control group less than 2.326 standard deviations. Also, for myxovirus having an HA titer of 1:640 or less, the protection is relatively constant among the challenge viruses tested.

It is necessary to note here that the Guaroa virus challenge was conducted in adult mice. While adult mice are not normally susceptible to Guaroa virus by the ip route, these mice harbored the ascites form of Sarcoma 180 which rendered the adult mice susceptible by this route. All other titrations in this group were in suckling mice.

In the second group of experiments, a series of myxoviruses were tested for their protective effect against one or both of two representative arboviruses. Results of this series are shown in Table 7. While no comparison of protection

is made between the different myxoviruses because of their different HA (and presumably infective) titers, it is apparent that the protective effect is not limited to B/Den/59. On the basis of these data it would appear that type A influenzas are generally lethal for mice, however, having a passage history in mice could partially account for this appearance.

The next group of experiments sought to compare the protective capacity of several myxoviruses and a synthetic double-stranded RNA (P:IC-dx) against challenge by several strains of WEE (Table 8). The 80 mg per cent P:IC inoculum represented a saturated solution at 5° C. While a significant protection was afforded by all agents used, NDV alone is apparently much more effective. The efficacy of NDV in the induction of protection against several arboviruses is shown in Table 9. (Ilheus and Louping Ill are very closely related to RSSE). The Sindbis LD_{50} in the NDV-protected group was less than 10^{-1} which accounts for the missed endpoint, however a protective index of over 2.5 million is undoubtedly significant.

The next series of experiments were designed to more clearly characterize the nature of the protection, using NDV and Sindbis viruses as the model system. Aliquots of NDV (E22) were inactivated by exposure to UV irradiation for varying lengths of time and resultant egg infectivity determined (Figs. 1 and 2). The ability to protect against Sindbis was determined for each irradiated sample. In addition, a matched control of comparable infective titer, achieved through appropriate dilution of an aliquot of an unirradiated sample,

was tested. Further, the 24 hour interferon titer in brains of mice receiving some of these agents was obtained through the courtesy of Dr. E. R. Borden, Developmental Virology Unit, NCDC. These data are included in Table 10 because they may contribute to the understanding of the protective mechanism.

With the irradiated samples, egg infectivity decreases by over 10 million-fold while the protective ability decreases roughly ten-fold. In contrast, a 500-fold decrease on infectivity achieved through dilution causes an almost exact corresponding drop in the protection. Beyond this dilution, no protection is demonstrated.

A final series of experiments designed to explore the duration of the NDV-induced protection was only partially successful due to a contamination problem encountered in the series. While the series established the presence of protection through the tenth day following NDV inoculation, quantitation of the protection was impossible. The results of this series are presented in Table 11. It should be noted that when NDV is given 24 hours after the challenge arbovirus, the mortality rate at every dilution through 10^{-5} is 100 per cent, demonstrating a pattern similar to numerous Sindbis titrations having an LD_{50} endpoint of $10^{-6.2 \pm 10^{0.5}}$.

However, when Sindbis challenge follows the myxovirus, even by only eight hours, protection is demonstrable. An undetermined proportion of the mortality rate <u>after</u> challenge was caused by a contaminant in the NDV inoculum (ranging from 0 to 50 per cent of the test animals per litter <u>before</u> challenge). Even so, there was no mortality among the 10^{-1} groups challenged eight hours and six days following NDV inoculation,

illustrating survival against over 10^5 LD_{50}'s of Sindbis at both these times. Even ten days following NDV no more than 50 per cent mortality occurred at the 10^{-1} dilution.

DISCUSSION

The protection of the mouse brain against arbovirus challenge has been investigated from the standpoint of its possible use in a model system for the study of safe viral oncolysis. Information gained from this investigation will be used to construct an hypothesis regarding the mechanism of protection.

The terms "activity" and "infectivity" are sometimes used synonymously in current literature. In this discussion, "infectivity" refers specifically to the ability to initiate infection. "Activity" refers to the presence or absence of any measurable effect of the virus. Thus the ability to initiate a protective effect (protectivity) may be active or inactive (nonfunctional) in a virus particle, as may the infectivity, the hemagglutination ability, or any measurable enzyme activity. "Inactivation" refers to the transition from active to inactive of any specified viral function. An "incomplete" particle lacks one or more components of a complete infectious virus particle and is necessarily noninfectious (by definition) but may or may not possess any other activity.

The magnitude of protection against ip challenge appears to be dependent upon several factors. As seen in Table 10, protection decreases with increasing dilution of NDV so that protection appears positively correlated with the inoculum as measured by the egg infective titer, or even by the HA titer as in Table 6. Although no exact relationship is obvious here, protection of over four logs is not seen unless

the egg infective titer approaches 10^8 or the HA titer is over 1:640.

A second factor affecting the magnitude of protection as seen from Table 9, is the particular arbovirus used to challenge, possibly a result either of different modes of entry into the CNS by various arboviruses or of different cellular susceptibilities within the CNS and its coverings.

The onset of protection is established within eight hours (Table 11), a finding consistant with that of Vilches and Hirst. (Appearance of the earliest demonstrable protection was not investigated although an assay of the interferon level in the brain three hours after NDV inoculation was already one-sixth the 24 hour peak value of 636 interferon units.)

The duration of protection (Table 11) was not definitely established due to a contaminant in the experiment. The exact magnitude of protection with time must be validated through further experimentation. Never-the-less this experiment does allow some comments concerning the duration of protection. Since each mouse was experiencing a double risk of mortality, one from the contaminant and one from the Sindbis challenge, no conclusion can be drawn from those who died. Survivors, however, withstood the double jeopardy, including the Sindbis challenge. The fact that there was no mortality at the 10^{-1} dilution when challenged six days following NDV, shows that considerable protection existed through that time. Sindbis virus under test conditions usually exhibited a titer of approximately $10^{6.5} LD_{50}$ and was shown in this experiment to be greater than 10^5. Thus, greater than five logs of protection

were present for six days and, as judged by the 50 per cent mortality at the 10^{-1} dilution among those challenged at ten days, at least five logs of protection persisted for a ten day period. Of course this experiment needs future verification.

The obvious first suspect as the protection mediator is interferon. The non-specificity of the protection, its induction by inocula consisting of noninfective virus and even by inocula of synthetic double-stranded RNA (Table 8), all strongly suggest that interferon plays a primary role in the protection. Table 5 shows that the amount of 24 hour interferon induction for P:IC-dx and B/Den/59 (passage E6A) is roughly equal and the degree of protection afforded by these two agents is within 0.4 logs (S.D. for each is 0.4). With most myxovirus samples, insufficient interferon titers were run to permit conclusive correlations with protective ability, egg infectivity or hemagglutination titer.

One series of experiments, however, does contribute information on both the mechanism of protection and its method of mediation. Interferon titrations conducted after inoculation of irradiated aliquots of NDV or matched infectivity samples in mice (Table 10) did demonstrate an association. Thirty seconds of UV irradiation decreased the protection to 40 per cent of the unirradiated sample (3.1 logs to 2.7 logs) and caused an almost identical drop in the interferon titer (210 to 87 units). Three hundred seconds of irradiation reduced the protection to about eight per cent of the original (to 2.0 logs) and to about 15 per cent of the original interferon titer (to 31 units). Judging from the irradiated samples, the protective effect may be attributable

to interferon. In the control inocula, with matched infectivity achieved through dilution, a 300-fold drop in the protective index (3.1 logs to 0.6 logs) is accompanied by a 125-fold drop in interferon induction (201 to 16). Figure 3, however, shows that the relationship between interferon titer and protective ability in this range is linear with a correlation coefficient of 1.00.

A major discrepancy does exist between the protective ability of the irradiated and diluted matched samples (Table 10). In UV-exposed samples, egg infectivity decreases by over ten million-fold over 300 seconds of irradiation while the protective ability decreases roughly ten-fold. In addition, during the first 30 seconds of irradiation, the number of infective particles decreases from $10^{8.0}$ to $10^{5.85}$, a drop of 99.3 per cent, yet protectivity decreases from $10^{3.1}$ to $10^{2.7}$, a drop of only 60 per cent. In contrast, a comparable drop in infectivity via dilution (99.3 per cent decrease) is accompanied by a drop of 99.6 per cent in protectivity. Thus, while protectivity (and interferon stimulation) shows a close relationship with the dilution of the inoculum, the number of infective particles does not seem to be involved in this relationship.

It has long been recognized that noninfectious virus can induce interferon. Prior to irradiation, the NDV inoculum contains 10^{8} infectious particles, no UV-inactivated noninfectious particles, and an unknown number of incomplete noninfectious particles. After 30 seconds of irradiation, the number of noninfectious particles has increased by 9.98×10^{7} particles. Meanwhile, the protectivity has decreased

by 60 per cent. It appears that protectivity cannot be reasonably associated with either numbers of infectious or UV-inactivated noninfectious particles. The remaining known component in the inocula which could be responsible for the protectivity is the quantity of incomplete virus. Thus, over the range of protection up to three logs, the data suggest that protection is primarily due to interferon which is induced by incomplete virus.

Another unanticipated interpretation indicated by the data from the investigation of the effect of UV irradiation is demonstrated in Figure 1. When the duration of UV exposure is plotted against the exponent of the egg infectivity titer, a fairly uniform curve is obtained which on a log scale is nearly a straight line (Fig. 2). Superimposed on Figures 1 and 2 is the line best fitting the experimental points, obtained through linear regression. This inactivation curve could be interpreted to mean that the inactivation rate is constantly changing (decelerating) or alternately that two or more factors are being inactivated at different rates. Unless irradiation conditions undergo change, no change in the inactivation rate should occur. During experimental inactivation, all observable factors (distance, agitation, temperature, etc.) remained constant. Furthermore, the output of the UV source does not decrease by 50 per cent in several months, thus the conclusion must be accepted that there are two (or more) factors undergoing different rates of inactivation. The solution to the dilemma as to whether there are (at least) two factors, one UV-sensitive (UVS) and the other UV-resistant (UVR) on the same virus particle or

whether there are two separate populations of viruses with different inactivation rates rests upon the assumption that each virus particle has only one mechanism of infection as opposed to two infection mechanisms, one UVR and UVS. As seen in Figure 1, both the UVS and UVR factors are measured by egg infectivity, indicating that two infective populations of virus must exist. Examples of NDV containing a proportion of a variant strain have been reported (59).

A similar finding regarding protectivity is evident in Figure 4. When the log of the protective index is plotted against the duration of UV exposure, the resultant curve of the loss of protectivity suggests that two (or more) protective factors are being inactivated by irradiation, one UVS and the other UVR. Additional evidence that protectivity is not directly associated with infectivity is the fact that the rate of inactivation of infectivity proceeds at such a tremendous rate as compared to the rate of inactivation of protectivity. This can be illustrated by comparing the log of the slope of the curve of protectivity inactivation (-4.2) with the log of the slope of protectivity inactivation (-0.7) over the first minute, or over the last four minutes (-0.8 as compared to -0.1).

The finding that protection against Sindbis challenge is directly proportional to the interferon titer (correlation coefficient = 1.00) supports the hypothesis that over this range of protection (up to a protective index of 3.1) a major portion of the protective effect is attributable to interferon. There is no evidence that extrapolation of this relationship to the observed protective index of $10^{6.4}$ would

accurately predict the number of interferon units (estimated at $10^{5.4}$) detectable by assay. It is possible that the amount of protection capable through the interferon mechanism falls short of the maximum protection demonstrated in this investigation and that a protective effect due to other phenomena occurs at high protective indexes.

The finding of two or more infective populations in the NDV is consistent with current literature. Since these populations vary in their UV-sensitivity it would not be surprizing that the incomplete particles also differ in the UV-sensitivity of their ability to induce interferon. Because the current understanding of interferon induction involves both infectious virus and UV-inactivated noninfectious virus, the suggestion that neither of these particle types is actually involved must be made as an hypothesis until confirmation by this or other investigators. The use of ic receptor-destroying enzyme could help determine whether the active attachment of virus particles to the cell is neccessary for interferon induction in vivo. Inactivation of myxovirus through other methods could help to define other necessary criteria for protection induction.

Another aspect of this problem which remains to be investigated is the degree of protection of nonneural tissues against arbovirus proliferation. Use of a "viscerotropic" strain of yellow fever virus might help to evaluate this aspect.

SUMMARY

The phenomenon of protection against arbovirus challenge by intracerebral myxovirus has been investigated. General findings include a rapid onset of protection and a magnitude of protection which can excede a protective index of 10^6, depending upon the myxovirus inoculum and the arbovirus used to challenge. Significant protection appears to remain as long as ten days after ic inoculation but exact quantitation has not been established. Over a defined range of protection, the amount of interferon produced is directly proportional to the protective index. Evidence is presented supporting the hypothesis that neither infectious virus nor UV-inactivated, noninfectious virus is responsible for interferon induction. Implicated instead is incomplete (non-irradiated, noninfectious) virus. The indication that the infective factor and the protective factor are both comprised of multiple populations is consistent with this hypothesis and with current literature.

Further investigation must establish whether or not this protection can be exploited to achieve safe viral oncolysis. The white Swiss mouse, Newcastle Disease virus and Sindbis virus seem to provide a reliable model system with which the numerous variables in oncolysis can be systematically explored.

TABLE 1

Incidence Of Oncolysis In Some Selected Tumor-Virus Experiments*

VIRUS	TUMOR** S180	MCI	E0771	RIDGEWAY	WAGNER
VEE	±	±	±	+	+
Semiliki		0	0	0	0
EEE	0	0	0	0	0
WEE	0	0	0	0	0
RSSE	+	+	+	+	-
Louping Ill	+	+	+	-	0
SLE	+	+	+	-	0
West Nile	+	+	+	+	+
Jap B	0	0	0	0	-
Yellow Fever	0	0	0	0	0
Ntaya	0	0	0	0	0
Bunyamwera	0	0	0	+	+
Bwamba	0	0	0	0	0
Passos I	0	0	0	0	0

KEY: +, oncolysis; 0, no oncolysis; ±, variable;
-, not reported

*Data summarized from references 5 and 6.
**S180, a sarcoma; MCI, a fibrosarcoma;
E0771, a mammary adenocarcinoma; Ridgeway and Wagner, both osteosarcomas

TABLE 2

Titer* OF RSSE Virus In Brain And Tumor After ip Inoculation Of 0.05cc Of A 10^{-3} Virus-brain Suspension in Mice**

DAYS	BRAIN	TUMOR (S180)
1	<1	4.50
2	2.50	7.00
3	4.25	8.50
4	4.50	8.25
5	6.50	8.50
6	8.50	8.50

*Titer expressed as log LD_{50}
**Taken from reference 8.

TABLE 3

Per Cent Of Viable Cells Remaining In S180 Tumors Following ip Inoculation With RSSE In Mice*

LD50's RSSE Injected	Per Cent Cells Remaining Viable On Day 1	3	4	5	6	7	8
10^5	42	0	-**	-	-	-	-
10^4	67	43	32	-	-	0	-
10^3	100	60	-	33	0	-	-
10^2	100	100	100	30	10	7	0
10^1	-	100	-	57	-	17	0

*Taken from reference 8.
**-, not done

TABLE 4

Susceptibility of Mouse Central-Nervous-System Cells to Infection as Shown by Fluorescent Antibodies*

Virus	Meninges or Ependyma or Both	Glia	Neurons
Poxviruses	+	0	0
Influenza:			
Standard strains	+	0	0
Neuroadapted strain (NWS)	+	+	+
Herpes Simplex	+	+	+
Reovirus	+	+	+
Arthropod-borne viruses:			
Group A:			
Sindbis	+	+	+
Venezuelan	+	0	+
Group B:			
West Nile	0	+	+
Murray Valley	0	+	+
Tick borne	0	+	+
Japanese	0	+	+
Ungrouped:			
Colorado Tick Fever	0	0	+
Rabies	0	0	+

*Condensed from reference 46.

TABLE 5

Agents Used in the Investigation of Protection against Arbovirus Challenge, with Infective Titer, Hemagglutination Titer, and Passage History

Myxovirus	Passage	HA Titer	EID_{50}	Interferon*
A/Swine/1976/31	E48	1:256		
A/PR8/34	F8/M593/EM0	1:1024		
B/Lee/40	F8/M137/E186	1:8		
B/Md/59	MK2/E1/MK1/E11	1:2048		
B/Md/59	MK2/E1/MK1/E12	1:160		
B/Md/59	MK2/E1/MK1/E13	1:160		
B/Md/59	MK2/E1/MK1/E14A	1:1280	$10^{7.0}$	
B/Md/59	MK2/E1/MK1/E14B	1:640	$10^{7.0}$	
B/Md/59	MK2/E1/MK1/E15B	1:2048		
B/Md/59	MK2/E1/MK1/E15B	1:1024		
B/Den/59	E5	1:80		
B/Den/59	E6A	1:640		64
B/Den/59	E6B	1:1024		
B/Den/59	E7	1:256	$10^{5.0}$	
B/Den/59	E8	1:128	$10^{4.0}$	
NDV	E21	1:160	$10^{7.0}$	636
NDV	E22A	1:1024		
NDV	E22B	1:2048	$10^{8.0}$	201
NDV	E23	1:512	$10^{7.7}$	152
P:IC-dx**				74

*Expressed as units of interferon/gm of brain, assayed by Dr. E. R. Borden on pooled suspensions of mouse brains harvested 24 hours after myxovirus inoculation.

**80 mg % solution of synthetic double-stranded RNA (polyinositic, polyaytidylic acid) in 80 mg % DEAE-dextran.

TABLE 6

Protective Effect of Various Passages of Influenza B/Den/59 given ic against Various Arbovirus Challenges ip in Suckling Mice.

Passage Designate	Titer HA	Titer EID_{50}	Challenge Arbovirus	Log Protection	Protection S. D.*
E6A	1:640	-	Langat	1.5	0.35
E7	1:256	10^5	Guaroa**	1.5	0.54
E7	1:256	10^5	Ilheus	1.4	0.24
E7	1:256	10^5	Louping Ill	1.4	0.30
E8	1:128	10^4	WEE (Fleming)	1.0	0.42
E6B	1:1024	-	Sindbis	4.0	0.41

*--Standard deviation of the protection, in logs
**--Test done on adult mice which, in order to create ip susceptibility, harbored the ascites form of Sarcoma 180

TABLE 7

Protective Effect of Various Myxoviruses ic against Langat or Sindbis Challenge ip.

Myxovirus	Titer HA	Previous Mouse Passage	Log Protection	Protection S.D.*	Challenge Arbovirus
NDV	1:2048	-	5.3	-	Sindbis
B/Den/59 (E6B)**	1:1024	-	4.3	0.41	Sindbis
B/Lee/40	1:8	+	1.4	0.32	Sindbis
A/PR8/34	1:1024	+	Lethal to mice		Sindbis
A/Swine/1976/31	1:256	+	Lethal to mice		Sindbis
B/Den/59 (E6A)**	1:640	-	1.5	0.35	Langat
B/Md/59	1:160	-	1.9	0.31	Langat
A/PR8/34	1:2560	+	Lethal to mice		Langat

*--Standard deviations of the protection, in logs
**--Also recorded in Table 6

TABLE 8

Protective Effect of Several Myxoviruses and a Synthetic Double-stranded RNA ic against Two WEE Strains Given ip

Interfering Agent	WEE Strain	Log Protection	Protection S. D.*
NDV (E20)	Fleming	2.8	-
B/Den/59 (E8)**	Fleming	1.0	0.42
NDV (E21)	558	3.8	0.33
P:IC-dx***	558	1.1	0.37

*--Standard deviation of the protection, in logs
**--Reported in Table 6
***--80 mg% solution of synthetic polyinositic, polycytidylic acid in 80 mg % DEAE--dextron

TABLE 9

Protective Effect of NDV Given ic against ic Challenge by Several Arboviruses

NDV Passage	Challenge Arbovirus	Log Protection	Protection S. D.*
E21**	WEE (558)	3.8	0.33
E22	Ilheus	4.3	0.29
E22	Louping Ill	3.3	0.33
E22	Sindbis	<6.4	-

*--Standard deviation of the protection, in logs.
**--Reported in Table 8.

TABLE 10

Effect of UV Irradiation and Dilution on the Protective Ability of ic NDV (E22) against ip Sindbis Challenge

NDV Treatment	Resultant EID_{50} Titer	Log Protection	Protection S. D.*	25 Hour Interferon**
none	$10^{8.0}$	3.1	.40	201
UV 30 sec,	$10^{5.3}$	2.7	.33	87
Dil $10^{-2.7}$	$10^{5.3}$	0.6	.30	16
UV 60 sec,	$10^{3.7}$	2.4	.42	nd***
Dil $10^{-4.3}$	$10^{3.7}$	0.3	.33	nd
UV 180 sec.	$10^{2.3}$	2.3	.33	nd
UV 300 sec.	$10^{0.7}$	2.0	.33	31
Dil $10^{-7.3}$	$10^{0.7}$	0.4	.33	8

*---Standard deviation of the protection, in logs
**---Units of interferon expressed as units/gm of brain. Done by Dr. E. R. Borden
***---Not done

TABLE 11

Percent Mortality among Mice* Challenged with Sindbis ip before and after ic NDV (E23)

Dilution of Sindbis	Percent mortality, per day after receiving NDV						
	-1	0 (8 hr.)	1	3	6	8	10
10^{-1}	100	0	43	67	0	43	50
10^{-2}	100	13	29	83	38	100	60
10^{-3}	100	100	14	0	50	67	20
10^{-4}	100	75	43	33	13	57	40
10^{-5}	100	0	0	33	38	29	25

*--Number of mice per litter ranged from 4 to 8

Figure 1. UV Inactivation of NDV Infectivity.

Infectivity remaining in aliquots of NDV with increasing exposure to UV irradiation. Infectivity is expressed as the logarithm of the 50 per cent egg infective dose and plotted on an arithmetic scale.

Figure 1

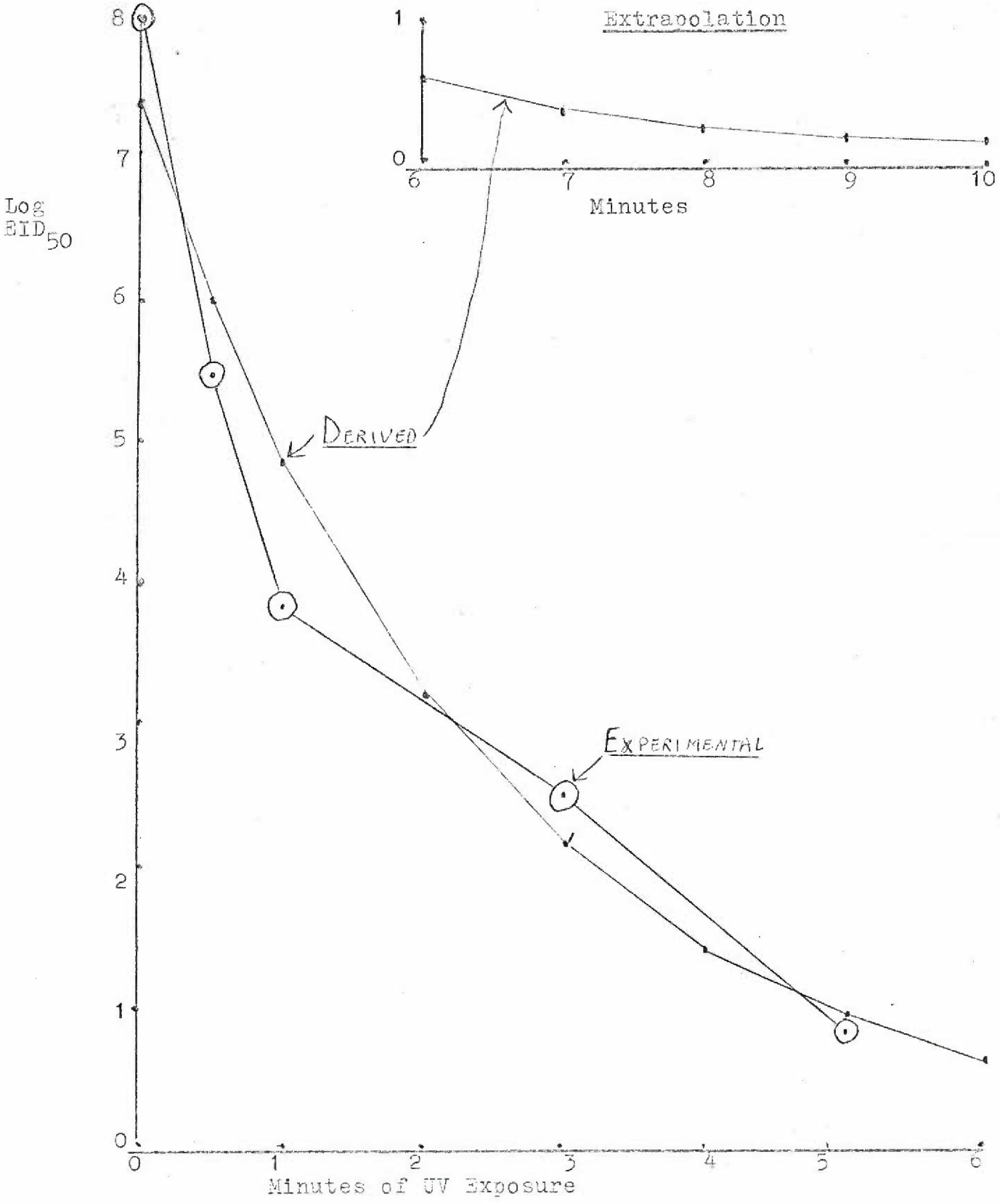

Extrapolation
1
0
6
7
8
9
10
Minutes
Log EID50
8
7
6
5
4
3
2
1
0
DERIVED
EXPERIMENTAL
0
1
2
3
4
5
6
Minutes of UV Exposure

Figure 2. UV Inactivation of NDV Infectivity

Infectivity remaining in aliquots of NDV with increasing exposure to UV irradiation. Infectivity is expressed as the logarithm of the 50 per cent egg infective dose and plotted on a logarithmic scale.

Figure 2

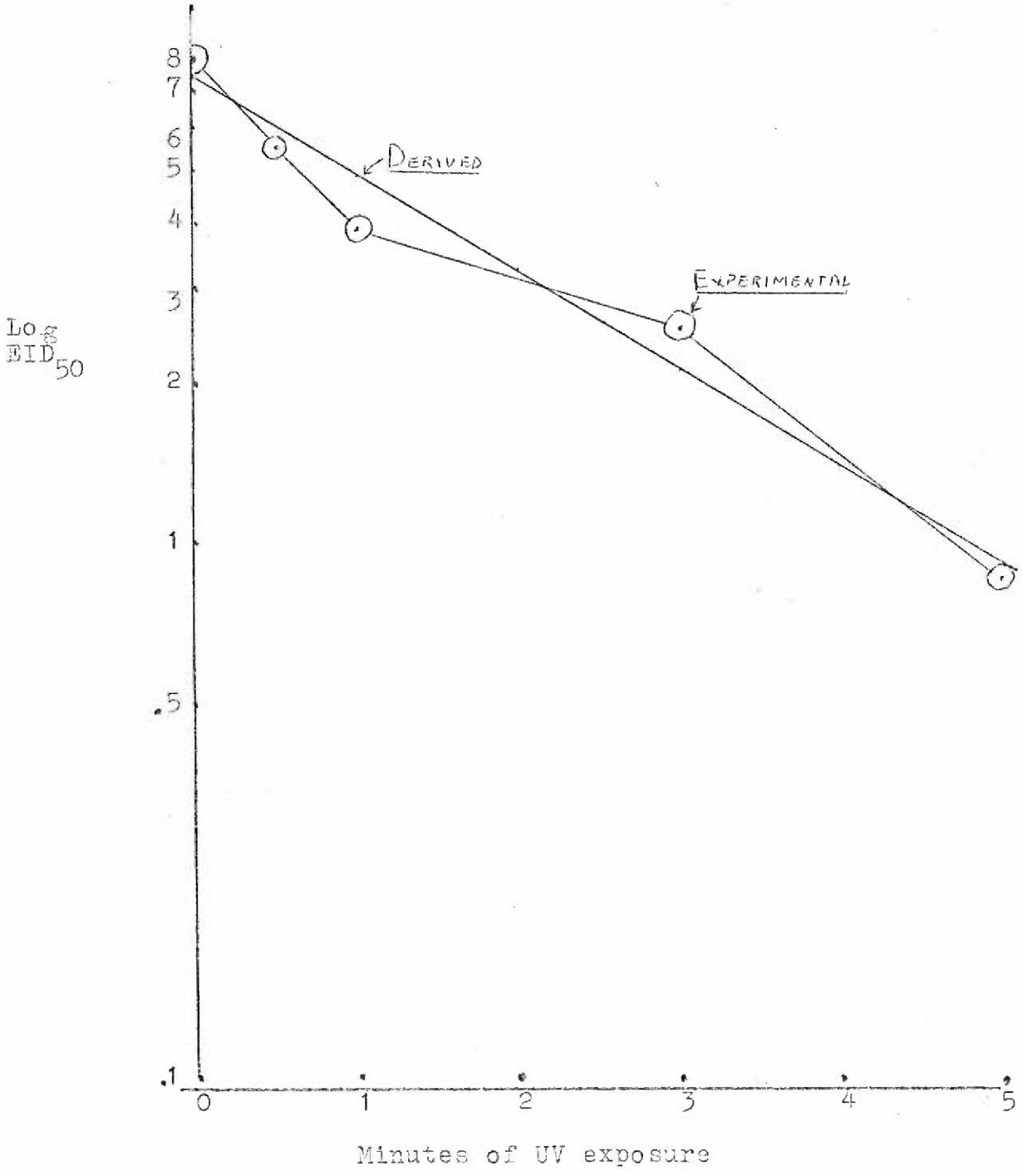

8
7
6
5
4
3
2
1
.5
.1
Log EID50
DERIVED
EXPERIMENTAL
0
1
2
3
4
5
Minutes of UV exposure

Figure 3. Relationship of Protective Index to Interferon Units

Correlation between the protective index obtained when ic NDV (E22) was used to protect against an ip Sindbis challenge, and the activity of interferon in the brains of mice twenty-four hours after ic NDV inoculation. Interferon assay was done by Dr. E. R. Borden.

Figure 3

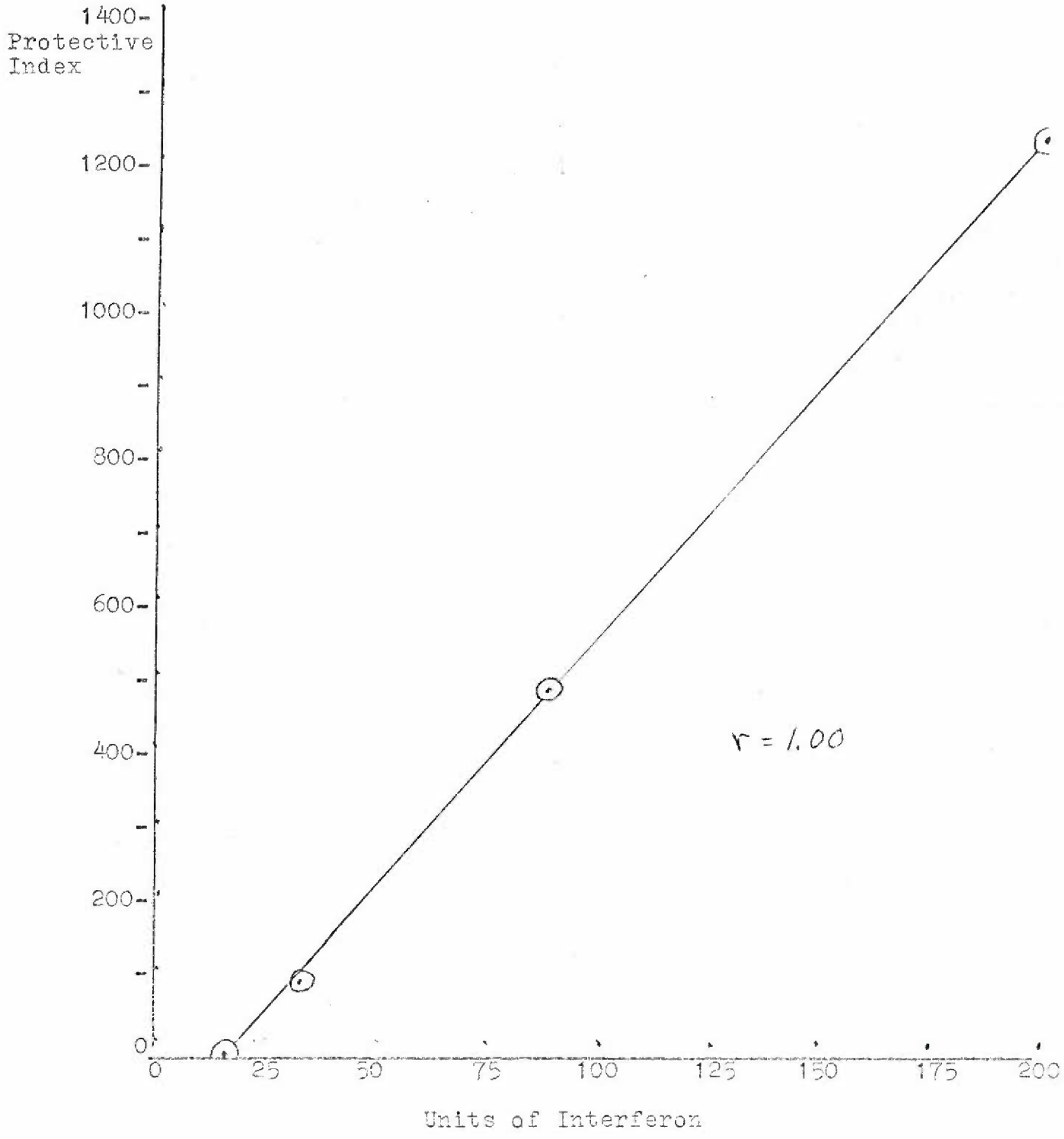
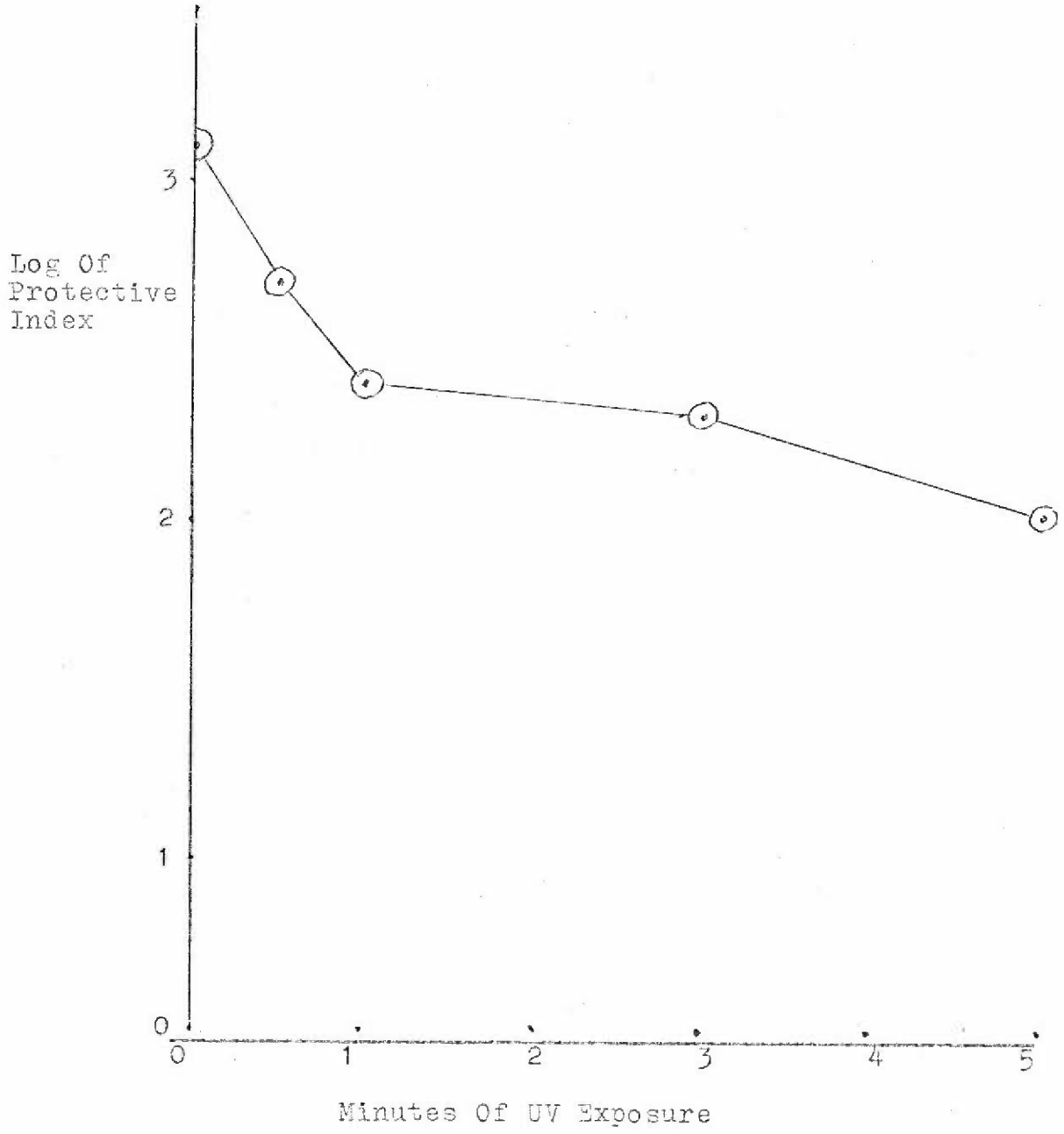

1400-
Protective
Index
1200-
1000-
800-
600-
400-
200-
0
0
25
50
75
100
125
150
175
200
r = 1.00
Units of Interferon

Figure 4. UV Inactivation of NDV Protectivity

Protective ability remaining in aliquots of NDV with increasing exposure to UV irradiation. Protectivity is expressed as the logarithm of the protective index obtained when ic NDV was used to protect against ip Sindbis challenge.

Figure 4



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