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Growth curves of herpes simplex virus in the mouse brain and their application to a study of possible relationships between herpes simplex and vaccinia viruses

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GROWTH CURVES OF HERPES SIMPLEX VIRUS IN THE MOUSE BRAIN AND THEIR APPLICATION TO A STUDY OF POSSIBLE RELATIONSHIPS BETWEEN HERPES SIMPLEX AND VACCINIA VIRUSES

by

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Introduction

The term herpes simplex, by common usage, refers to a recurrent illness usually characterized by a single blister, or small group of blisters, about the mouth. To most people this condition is familiar as the annoying, yet generally benign "cold sore" or "fever blister" which refuses to respond to treatment in a satisfactory manner, but regresses and disappears in due course. Fortunately, this most frequent manifestation of infection with the virus of herpes simplex is relatively trivial. Other less common clinical pictures range from painful lesions involving the mouth and pharynx, to diffuse often severe eruptions of the skin, and occasionally fatal infections (73, 78). The virus affects the genitalia, and not uncommonly the cornea, and may involve the central nervous system where it gives rise to less well defined symptoms. Cases have also been reported of involvement of the internal organs (56). Burnet (17) has stated that except for the common cold, herpes simplex is the most frequent viral disease of man.

Although recovery from many viral diseases results in an immunity which lasts for years, it is well known that in herpes simplex there is no protection from subsequent attacks. Patients frequently undergo ten or more repeated episodes yearly with little reduction in severity until late in life.

To explain reinfections, which seem to occur without re-inoculation, it has been suggested that the source of the virus is intrinsic, with the virus generally persisting once the host has become infected (17). In this way, clinical symptoms can recur following any of a large number of "trigger" stimuli, such as overexposure to sunlight, emotional upsets, and the like. The manner of activation of the latent virus is unknown, but may be considered to depend upon certain " 'predisposing factors' which tend to lower the resistance of the . . . . . tissues" (41).

Of the current forms of treatment, none have been entirely satisfactory. For the more severe herpes simplex infections, therapy is entirely symptomatic (41, 73). With the more common recurrent type of lesion, treatment is usually not required, nor is it generally effective when applied. Although there have been several reports to the contrary, the broad-spectrum antibiotics appear to have little value except in limiting secondary infection (27). Some consider the sole effective treatment of the recurrent herpetic lesion to be the use of repeated smallpox vaccinations.

The use of this method in the suppression of recurrent herpetic infection appears to have developed as a result of the widespread adoption of vaccination for the prevention of smallpox. Jenner in 1804 devoted a lengthy report to a consideration of cases in which recurrent attacks disappeared or

lessened in frequency following vaccination, and he expressed his belief that this was due to the "constitutional reaction" produced by the vaccine (63). In addition, he noted that vaccination with calf lymph might fail to "take" in a subject with herpetic vesicles, a point which Roxburgh (65), in 1927, considered to be quite interesting inasmuch as such cases were not heard of in more recent years. Certain other clinical records have revealed that few people (2 cases out of 3000) suffer from concomitant herpes and smallpox (65).

The methods which have developed from these and other observations for treating herpes simplex infections by smallpox vaccination are all very much alike. Briefly, they call for the patient to submit to a course of from 2 to 12 vaccinations at weekly or bimonthly intervals, with the punctures performed either directly over the affected area, when cosmetic effects are not important, or on some other part of the body. Freund (32) treated 7 cases of genital and extragenital herpes in this way and reported freedom from recurrences for at least seven months. Minami and Ohmichi (57) followed 10 cases of herpes genitalis over a period of two years with 7 out of 10 patients showing improvement or cure. In 1934 Wise and Sulzberger (87) reported satisfactory results with repeated vaccinations in some, but by no means all, of their patients. Similarly, Foster and Abshier (29) observed only 5 recurrences

of symptoms in 35 patients at the end of two years. Cases of corneal herpes (24), ulcers of the mouth and tongue (42, 64, 89), and additional cases of cutaneous herpes (4, 40) have been the more recent to appear among reports in which favorable results were obtained.

To balance this rather enthusiastic picture, a number of clinical reports have dealt with the apparent failure of smallpox vaccine to protect (11, 49). Although these may represent only a small fraction of the actual failures encountered in general practice, they are represented in a larger sense by the general attitude of some reviewers that vaccination is of little value (9, 13, 46).

Blank (10) and others (16, 25, 50) have questioned the identity of the infecting virus in certain cases attributed to herpes simplex. They found that many of the cases of recurrent stomatitis (aphthous ulcers) are not due to herpes simplex as formerly believed, but that the cause of this condition in most patients is obscure. In this respect, Ronchese (64) has presented the case of a woman suffering from this condition who showed decided improvement after three inoculations against smallpox. This interesting response of a presumably non-herpetic condition to smallpox vaccine has not been commented upon by other observers. Grace (42) has presented a similar case.

Blank and Brody (11) in discussing the apparent clinical successes obtainable in treating recurrent herpes with smallpox

vaccination suggest that this may be in reality nothing more than "suggestive therapy". They were able to bring rapid relief to 10 individuals by alleviating, through psychiatric treatment, feelings of shame or guilt associated with the symptoms. Blank (9) feels that in view of the absence of any detailed clinical studies using a control, such as heat-inactivated calf lymph on alternate cases, a proper evaluation of vaccinial treatment cannot be made. Baldridge and Sternberg (5) add that the frequency of spontaneous remission of herpetic lesions makes it unwise to venture any opinion favoring the use of smallpox vaccine for the suppression of recurrent herpes simplex infection.

It has been claimed that the effect of successive smallpox vaccinations is to raise the herpes simplex antibody titer by injection of a related virus (29). Unfortunately, as Baldridge and Sternberg (5) point out, it has been repeatedly shown that individuals subject to herpes generally possess high antibody levels at all times. Nevertheless, some success has been obtained in alleviating recurrent herpes through the use of formolinized herpes virus (13, 31) and the autogenous inoculation of herpetic vesicle fluid (51, 72).

In an attempt to explain the beneficial effects of smallpox vaccination on recurrent herpes simplex infections, early investigators turned from clinical trials to an examination of possible immunologic relationships. Gildemeister and Herzberg

in 1925 and 1927 produced evidence that when the rabbit cornea and guinea pig foot pad were immunized against herpes virus, these areas also showed a partial immunity to variola virus. A definite viral relationship was therefore claimed, which was further supported by in vitro neutralization experiments based on the inactivation of variola virus by herpes antiserum prepared in the rabbit. Similarly, these workers found that animals immunized with variola virus developed only mild reactions upon injection of herpes. These observations were confirmed and extended by St. Zurukzoglu (66), whose paper appeared early in 1927 along with the second paper of Gildemeister and Herzberg. Working with vaccinia virus in the place of variola, he indicated that a distinct partial immunity did exist between the two viruses. However, vaccinia immunized guinea pigs were less immune to herpes virus than herpes immunized guinea pigs were to vaccinia virus. Freund and Heymann (33) have provided confirmation of these findings.

Since the appearance of the above papers between 1925 and 1927, no new or confirmatory evidence has been advanced. Several teams have repeated the cross immunity and serum neutralization tests, but with completely negative results. The work of Bedson and Bland (8) is particularly revealing in that they have taken into account the blocking effect that a prior inflammatory skin reaction may have on a virus applied to the same area. First, they inoculated herpes or vaccinia virus

on the shaved skin of guinea pigs, and after allowing 4 to 6 weeks for recovery, tested the animals for immunity with the same virus. If the results of this test were satisfactory, the second virus was titrated on the skin, being careful that the highest test dilutions were placed over the area of the strongest "take" given by the immunizing virus. Thus, the experiments have been weighted in favor of the findings of Gildemeister and Herzberg. In all cases, nevertheless, the lesions resulting from the challenge virus differed little from the controls.

Serum neutralization tests were also repeated. Rabbit antiserum diluted 1:2 was mixed with varying dilutions of homologous or heterologous virus, allowed to interact for one hour at room temperature, and then inoculated into guinea pigs by scarification. Again there was no cross protection. The authors conclude, consequently, that the viruses of herpes febralis (herpes simplex) and vaccinia are unrelated. Commenting on the original observations of Gildemeister and Herzberg, Bedson and Bland write:

> "Had the work of Gildemeister and Herzberg consisted only of cross-protection experiments in the animal, frequently entailing superimposition of inoculations, one would have been less ready to pay heed to their conclusions. But they reported in addition neutralization experiments with specific sera which bore out their <u>in vivo</u> findings."

They note that this has not been the experience of others.

The techniques of Bedson and Bland have been modified by

several groups with essentially the same results. Perdrau (63), and later Thompson and Buchbinder (85), used rabbits known to be immune to either herpes simplex or vaccinia by intracerebral challenge. Introduction of the heterologous virus by dermal or intracerebral routes failes to reveal any immunity whatsoever. Olitsky and Long (61) reported similar findings in guinea pigs, and Kanazawa (48), in mice. In the latter case both fresh and phenolized herpes virus and vaccinia virus were used for immunization. As before, no cross immunity was detected.

To complete the immunologic investigations of a possible relationship between smallpox vaccination and herpetic infection, Smith (79) attempted specific and cross antibody absorptions with the viruses of vaccinia and herpes. Only the specific reaction could be demonstrated. Lastly, Gildemeister and Ahlfeld (37) using passive protection tests, showed that a subcutaneous injection of 0.5 ml smallpox vaccine did not protect mice against simultaneous cutaneous injection of herpes virus. The use of complement fixation tests in this problem has not appeared in the literature.

The preceding investigations have constituted a type carried out up to 1938. More recently, a number of different experiments have been devised which point to more fundamental differences in the behavior of the two viruses; that is, in

their intracellular position. These experiments, first of all, may be considered as logically following the work of Levaditi and Reinié (54) and Levaditi (53) who were able to pass both vaccinia and herpes at the same time intracerebrally in rabbits. They have stated that the two viruses "can exist together, indefinitely and symbiotically in the brain and cord of rabbits . . . . . This has been established during 18 consecutive passages." Anderson (3), a year later, following the original suggestion of Syverton and Berry (83, 84), showed that herpes simplex and vaccinial inclusions could be found in the same cells of the chick chorioallantoic membrane. These doubly infected cells were located with difficulty and not with any degree of regularity, as was emphasized by Syverton (82) who could find only one such cell after making numerous serial sections of the virus-inoculated conjunctival sacs of three rabbits. Typically, the irregular intranuclear inclusion bodies characteristic of cells infected with herpes simplex could be found along with the larger, more spherical intracytoplasmic inclusions characteristic of vaccinia. The possibilities of a mistaken interpretation caused by one cell overlying another were fully appreciated, and care was taken to minimize the likelihood of this error. Gaylord et al. (35), Bang (6) and others, have shown the vaccinial inclusions to be aggregates of elementary bodies.

Although the inclusions of herpes simplex are nuclear in location, evidence adduced by centrifugation studies on the

separation of structural units of cells has indicated that the virus is more richly associated with the cytoplasm (30). Francis and Kurtz (30) feel that there is no selective binding of virus to nuclei or to nuclear nucleoprotein, and with Bang (6), believe that the inclusions are not herpes virus. This view is also taken by Ackermann and Kurtz (1) who employed cell fractionation technic in their work.

Despite the above statements, however, there is convincing evidence that during the early stages of development the herpes inclusions do represent an aggregation of actual viral material. As Crouse and her coworkers (23) have pointed out (confirmed by Wollman and Baker (88), it is possible to demonstrate a positive Feulgen reaction on these inclusions, showing that they contain desoxyribose nucleotides. Furthermore, Scott et al. (74), in a recent paper, have shown by histological methods that during the first 10 hours of infection of rabbit corneal cells, a series of changes occurs in the cell nucleus leading to the development of a rare, shrunken, type A inclusion body. The more usual "fully formed" inclusion which appears at about 12 hours, is apparently only a stage in viral development, although probably the final one. Both Francis and Kurtz (30) and Ackermann and Kurtz (1) are criticized for not taking this sequence into account, and for examining the inclusion body for virus at 48 hours when presumably no virus was left. The inclusion can therefore be regarded as a

Feulgen negative skeleton just prior to and after the release of virus from the cell, whereas early in the growth cycle it is Feulgen positive. Confirmation of these observations is provided first by Gray and Scott (43), who have indicated by means of cell fractionation that virus is associated with the nucleus at a definite stage in the growth cycle; secondly, Morgan et al. (60), with the electron microscope, have indicated the presence of elementary bodies in the nuclear inclusions.

Briefly summarized, the development of herpes simplex inclusions, and virus, in the nucleus, and vaccinial inclusions, and virus, in the cytoplasm, serves to emphasize a major difference between the two viruses, which may be added to the immunological differences already mentioned.

Growth curves have been determined for a large number of viruses in animals, tissue culture, and developing chick embryos. Practical applications of such growth curves have been limited, however, but have yielded valuable information (55). For example, the effect of a variety of chemicals on the growth of poliomyelitis virus in tissue culture has been described by Brown (15), and Briody and Stannard (14) have tested the effects of the acridine compound, proflavine, on the growth of mumps, influenzal, Newcastle disease and vaccinial viruses in the chick embryo. Burnet and Lush (18) and Shaffer and Enders (70) have noted the effects of immune serum on herpes simplex virus multiplication in the chick embryo,

and Cheever and Daikos (22) have extended this to include the effect of gamma globulin.

Presentation of the Problem

In examining the relationship of herpes simplex and vaccinial viruses, the likely assumption is made that the possible suppressing effect of vaccinia is a result of viral interference. Indirectly, this assumption has been subjected to extensive study in the past, through experiments dealing with five of the six hypotheses for interference as presented by Henle (45). However, one basic question remains unanswered - namely, can vaccinia virus or its products actively destroy herpes virus? Rather than design an experiment to answer this question, it was felt to be more logical to examine as many of the factors involved in interference at one time as possible. To accomplish this purpose, the technic of the virus growth curve was chosen, since it offers a highly sensitive procedure that is very likely to permit the detection of subtle changes.

Materials and Methods

Viruses. The herpes simplex virus employed throughout this study was a mouse adapted HF strain * with a uniformly high neurotropic infectivity. It was originally isolated by Flexner and Amos (42) from the fresh lip vesicle of a subject very prone to attacks of febrile herpes, and has since been maintained for more than 25 years in experimental animals. The virus as received was reconstituted from the lyophilized state with 0.85 per cent NaCl and inoculated in 0.03 ml quantities into the brains of Harvard strain Swiss albino mice. These animals developed signs of an encephalitis and died within from two to three days. The brains were aseptically removed, pooled, and from them an emulsion prepared that was used for further mouse passages. In the present series of experiments the 19th to 33rd mouse passages were used. The infectivity titer of these passages, as measured by intracerebral test, was between $10^{7.9}$ and $10^{8.4}$ MPLD (See page 17).

The vaccinial viruses employed were of two types: one, for animal immunization, was the standard vaccine preparation of the Massachusetts Department of Public Health, Division of Biologic Laboratories. Its history and origin are uncertain, but it appears to be a mixture of two strains of vaccinial

* Obtained from Dr. Howard B. Slavin as the 337th passage in mouse brain in his laboratory. It was lyophilized on June 20, 1951. The designation HF used throughout this paper refers to this strain of herpes simplex virus.

virus obtained respectively in 1920 and 1947 from the New York City Board of Health. The second virus, used for challenge, was the Levaditi (L) strain*. It was lethal for 3- to 5-week old mice by the intracerebral route of inoculation, killing with an MPLD titer of $10^{4.3}$ to $10^{5.3}$ within five to eight days. Experiments were performed with brain suspensions representing the 3rd mouse passage.

Preservation of viruses. In view of the pronounced instability of herpes simplex virus in physiological saline solution and the difficulty of maintaining material of a constant potency, no effort was made to preserve a fluid brain pool of the HF strain. Rather, the samples of virus employed in individual experiments were derived directly from fresh brain passages. Seed virus for these passages consisted of brains which had been stored in paraffin (Parafilm) sealed Petri dishes at - 40 C.

The L strain of vaccinial virus was stored at - 25 C and retained uniform activity as a 10 per cent brain suspension in 0.01 M phosphate buffered saline solution at pH 7.2. Samples of virus were dispensed in 2 ml quantities into screw cap tubes and frozen.

Assays for virus. The HF strain was assayed in the following manner. Suspensions of infected mouse brain were prepared by grinding fresh tissue in a chilled mortar with sterile

* Received from Dr. B. A. Briody as a 1:2 dilution of (?) per cent mouse brain in 50 per cent glycerol.

alundum and a small quantity of gelatin saline solution*. Sufficient diluent was added to give either a 10 per cent or 31.6 per cent brain suspension by weight (the latter concentration to permit the detection of virus in the low concentration of $10^{1.5}$ MPLD), and the material centrifuged in the cold (2-4 C) at 2000 rpm for 30 minutes (950 RCF (g) with an International No. 249 head) or 3000 rpm (2000 RCF (g))for from 45 to 60 minutes. When it was important to have the supernatant fluids relatively cell free, as for example in the work dealing with growth curves, the higher speed was always employed. The supernatant fluids thus obtained which represented the virus preparations, were tested for bacterial sterility. No noticeable bacterial growth took place either here or in mice receiving this material and it was felt unnecessary to incorporate antibiotics into the inocula. The quantitative measurements of virus were carried out in Harvard strain Swiss albino mice approximately 3 weeks old. Groups of five animals were inoculated intracerebrally, without ether anaesthesia, with 0.03 ml of serial 10-fold dilutions of virus. They were then observed daily for ten days for the occurrence of death. Animals dying within 24 hours were considered as nonspecific deaths and were excluded from the calculations.

Assays of vaccinial virus activity with the L strain

* Isotonic phosphate buffer solution (86) with 0.5 per cent gelatin (Difco). This diluent is used throughout this work.

were carried out in essentially the same manner as described, with the exception that the diluent did not contain gelatin. Suspensions were centrifuged only at 2000 rpm for 30 minutes.

Analysis of data. Estimations of infectious titers were made according to the method of maximum likelihood (97), using the tables of Hoskins (47) and Prescott (61a). Results are expressed as the most probable lethal dose of virus (MPLD per ml of inoculum). These were calculated from the distribution of deaths in three groups of five mice, each of which received successively higher serial dilutions of virus, usually 10-fold. This method offers several advantages over the customary Reed and Muench (62) determination of the 50-per cent end point when decimal dilutions are employed, most important of which is avoidance of the error inherent in the "accumulation method" of analysis. In addition, an approximate value may be obtained in those instances in which fewer than half the mice die in the lowest viral dilutions. For deaths among a small group of animals, this may afford a more suitable analysis than is obtained with other methods.

The standard error of the log of the MPLD was calculated by an amplification of the methods of Fisher (27a). Using 10-fold serial dilutions, five mice per point, the standard error with 95% confidence limits is 0.46. The error is less in those cases when the first dilution is 1/2 log. The minimum difference to which significance has been ascribed in the present experiments is $\pm$ 1/2 log.

Growth curves. Groups of mice 3 or 4 weeks old were incculated intracerebrally with HF virus in 10^{-1}, 10^{-3} and 10^{-5} suspensions. Smaller groups of 5 or 6 mice were killed with chloroform after various intervals following inoculation and their brains pooled for titration. These were carried out immediately without waiting for the remaining animals to be sacrificed.

Technic of vaccinial immunization. Suckling mice, 3 days old, were vaccinated on the skin of the back with vaccinial virus (Lot Nos. 310, 316, 319, 320 and 324)*. An amount of viral suspension estimated at 0.004 to 0.005 ml (on the basis of 4 or 5 animal applications per tube) was applied to the back of each animal, and through this material the back was lightly scarified 20 to 25 times with the tip of a sharp dissecting needle. This was sufficient to produce a deep red flush, but not heavy enough to break through the skin. The mortality rate with this procedure lay between 15 and 20 per cent, the majority of deaths occurring within two weeks. In certain instances, approximately 24 days after immunization, and 4 days before the use of these mice in experiments, a booster dose of 0.25 to 0.5 ml of a 10^{-2} suspension of vaccinial virus was administered intraabdominally. In one experiment this dose was repeated one hour prior to their use. The mice used as controls for those immunized were derived from litters born on the same day.

* Vaccination of younger animals (several hours old) gave a slightly poorer degree of protection as indicated by subsequent challenge.

Results

Several attempts were made to find a simple and direct means of estimating the concentration of HF virus in tissue preparations. These included various agglutination technics which had been successfully applied to the study of a variety of viruses, but never clearly established as functioning in systems involving herpes simplex.

Hemagglutination tests. According to the recent report by Geller et al. (36), five egg adapted strains of herpes simplex virus failed to agglutinate the red cells of several different animal types when examined by direct and indirect tests (See below). This is contrary to the earlier experience of Moolten and his associates (59) who consistently found the virus to cause autohemagglutination of patients' cells in vivo and indirect hemagglutination of cells sensitized with herpes simplex virus in vitro. While these latter observations were being made, experiments were underway by the author offering additional evidence of the nonhemagglutinating nature of herpes simplex virus. The exact procedures followed are outlined here.

Human Group O and fowl red blood cells were examined at room temperature and at 37 C for agglutination in the presence of serial twofold dilutions of a potent HF virus suspension. The red cell concentrations were varied between 0.25 and 2.0 per cent (final concentration) and the pH maintained between

7.1 and 7.2. Under these conditions no agglutination occurred, either as gross clumping of the cells or as a Salk (67) pattern. Tests were performed on a lucite plate (68) as well as in standard Wassermann tubes.

The indirect test was conducted by exposing red blood cells to concentrated HF virus for 1½ hours, washing the cells three times, and then adding them to serial dilutions of rabbit HF immune serum. There was no agglutination, either at room temperature or at 37 C.

In a third series of experiments, human Group O cells were treated with tannic acid according to the method of Boyden (12). These cells were then used in repeating the preceding indirect agglutination test. The results, as before, were entirely negative.

Liver cell agglutination tests. A further series of experiments was carried out, employing a preparation of cell nuclei in the place of erythrocytes. Fresh rabbit liver was prepared by the salt extraction method of Mirsky and Pollister (58) so as to yield a concentration of nuclei estimated by them to comprise more than half of the cell mass. Exposure of this material as a 0.5 per cent suspension to an equal quantity of serially diluted virus resulted in no noticeable reaction. The indirect agglutination test was not run with liver nuclei.

Developmental Pattern in the Mouse Brain

Growth curves for the HF strain of herpes simplex virus in the brain of the mouse have been reported by Burnet and

Lush (19) and Cheever and Daikos (22). However, their data are incomplete and do not lend themselves to a critical analysis or to the purpose of this paper. The following work was carried out to establish a more precise pattern of virus development.

The effect of time and speed of centrifugation on the pattern of virus growth. Recovery of significant quantities of virus during the latent phase of growth in mice has been reported for equine encephalomyelitis virus (71), Newcastle disease and influenza viruses (21), and the Lansing strain of poliomyelitis virus (2). The factors responsible for this recovery have not been definitely established. It was therefore desirable to examine closely the early phase of the growth cycle before determining the complete pattern of HF multiplication.

Accordingly, a series of experiments was conducted with the premise that certain characteristics of the inoculum are responsible for the failure of virus to disappear during this phase of growth. A 10 per cent virus suspension was centrifuged at 2000 rpm for 30 minutes. The slightly turbid supernatant fluid was separated, a sample set aside (Supernate I), and the remaining fluid recentrifuged at 3000 rpm for from 30 to 60 minutes. After removing a small sample of this supernate by pipette (Supernate II), the remainder of the fluid was decanted and discarded, and the sediment redispersed without further treatment and made up to the original volume with gelatin saline

solution (Sediment). Thus, Supernate I represents the usual inoculum which has been separated into a supernate (Supernate II) and sediment (Sediment). The growth curves obtained following the intracerebral inoculation of each of these preparations into mice are given in Figure 1 and Table I.

It is evident from an inspection of Figure 1 that after inoculating the animals with Sediment, the concentration of recoverable virus decreases at a much slower rate than in the case of either of the supernates. At the end of 3½ hours the level of recoverable virus is as great as in the case of Supernate I, while that resulting from inoculation with Supernate II has decreased almost to zero. From this we see that the Sediment contains considerable virus present in such a state, that after inoculation it is recoverable during that portion of the latent period which shows up as the lowest point on the growth curve. It may very well be that the failure to remove this sediment from the usual growth curve inoculum* is to some unknown extent responsible for the recovery of virus during the latent period of the growth cycle.

Further, the curve obtained with Supernate II (Figure 1) shows the presence of a consistently lower titer than that obtained with Supernate I, and a somewhat quicker loss of recoverable virus at the start of the cycle. This is more clearly shown in Figure 2, where Supernate I is represented by the upper

* It has been customary in virus work to clarify inocula by low speed centrifugation (2000 rpm for about 30 minutes).

curve and Supernate II by the lower. Since the inocula which were used to obtain the two curves differ only by the removal of cellular debris, one would expect that the curve obtained by inoculation with Supernate II would be lower.

The differences observed by varying the speed of centrifugation are tabulated in Table II, and seen to advantage in Figure 2. The two curves illustrated differ not only in that the inocula used were centrifuged at different speeds (2000 and 3000 rpm), but also in that the brain pools used to determine the points were also centrifuged at the same speeds used to prepare the respective inocula. As mentioned before, the more highly clarified inoculum yields a curve showing rapid loss of recoverable virus; about 99 per cent within one hour. The other curve, obtained with an inoculum containing sediment, shows only a 50 per cent loss in the same time. From one to four hours after inoculation, however, both curves show approximately the same rates of loss of recoverable virus and from four hours on, the same rates of gain. Consequently, the differences in recoverable activity that appear one hour after injecting virus remain constant throughout the growth period. This difference averages about 1 log unit. An increase in the speed of centrifugation, therefore, will depress all points on the growth curve, causing a downward displacement.* Possibly,

*In this experiment, as well as in others, a separate pipette was used in preparing each dilution. This avoids the possibility of tissue particles adhering to the glass and carrying over into higher dilutions. Such an error has been observed here, and by others (55, 80), to result in abnormally high titers. Despite this precaution, however, MPLD titers upwards of $10^{11.9}$ have been obtained for 10^{-1} brain suspensions clarified at 2000 rpm for 5 minutes.

this may represent nothing more than the effect of a simple decrease in the size of the inoculum, such as would result from the removal of potentially infective cellular debris.

Growth curves and the effect of varying dosage. In a series of six experiments, the growth curves were determined for three different concentrations of HF virus. No more than two of the three concentrations were run at one time, but the reproducibility of the curves within $\pm$ 0.5 log units for nearly all points, permits a rough comparison of the three. This has been done graphically in Figure 3 for 10^{-1}, 10^{-3} and 10^{-5} suspensions, containing, respectively, $10^{7.8}$, $10^{6.4}$ and $10^{4.4}$ MPLD per ml. Table III presents the crude data. The curves presented are typical values of these experiments.

Examination of the data reveals a cycle of development in the mouse brain conforming to the general picture of multiplication for a large number of animal (2, 21, 71) and bacterial (55) viruses. During the first hour or two following inoculation there is a rapid drop in recoverable virus. This is followed by a variable latent period, dependent upon the concentration of virus originally injected, during which time little or no virus is detectable. A sharp rise between the 4th and 6th hours of the 10^{-1} curve, the 7th or 8th hour of the 10^{-3} curve, and the 11th hour of the 10^{-5} curve suggests the advent of the cell "burst"* and rapid increase in the number of virus

* The term "burst" is used here in referring to a sudden sharp increase in virus concentration. The mechanism may be similar to that described for bacterial viruses (55).

particles. The period of rapid multiplication may proceed in a stepwise fashion, since, at the end of the latent period, there appear to be two or three marked increases in activity at intervals of about six hours. In some instances these steps fall within the limits of normal variation ($\pm$ 2 S.E. = $\pm$ 50% of one log unit). The remaining steps, particularly with the 10 per cent inoculum, are constant enough to suggest that they are real.

From an inspection of the curves, it is quite apparent that they are very much alike. In addition to stepwise increases they have similar initial slopes, thus suggesting a uniform rate of virus multiplication independent of the size of the inoculum. However, this similarity is lost as the virus concentration approaches a maximum. Presumably the number of cells not infected becomes a limiting factor in further incremental growth. This is best illustrated by the data for the 10^{-5} curve of Figure 3. In this case, the rate of production of virus decreases at about 30 hours.

The relation of survival time to dose. The time at which the test animals can be expected to die is roughly predictable for a given inoculum if the time at which a maximal titer is reached in the brain is known. Thus, with a 10 per cent inoculum, this point is reached at about 15 hours, and death generally follows at from 11 to 13 hours later. With the more dilute inocula a maximal titer is reached at about 24 hours (10^{-3} dose) and 40 hours (10^{-5} dose) and death follows

about 20 hours after maximum titers are attained; that is, at 2 days and 2½ days respectively. Concentrated inocula do not seem to follow the pattern set by the diluted inocula, in that they produce death very rapidly after reaching the maximal titer. This perhaps reflects the overwhelming type of infection which is induced by the heavy inoculum.

The Relationship of Herpes Simplex and Vaccinia Viruses

Serological and immunological technics have been employed in the past in attempts to relate herpes simplex and vaccinia viruses. The results of these tests have been essentially negative. Since clinical observations suggest that herpes and vaccinia do possess some factor in common, it is perhaps to be found on a possibly more subtle level than has yet been considered. Changes in the reproducible pattern of HF virus growth have been used as a sensitive means of detecting some hypothetical altering factor in vaccinia virus.

The growth curve of herpes simplex virus in mice immunized against vaccinia. Under clinical conditions the use of vaccination to suppress recurrent herpes infection generally results in a good immunity against vaccinia virus. The symptoms of recurrent herpes frequently disappear as this immunity develops, and they do not reappear - or do so only in a mild fashion - for long periods of time after the disappearance of circulating vaccinial antibody. These conditions relating to vaccinial immunity were readily simulated in mice, and their effects on the growth curve of herpes simplex virus determined.

Mice in the experimental group were vaccinated once at the age of 3 days. A small group of these animals resisted an intracerebral challenge of vaccinia L virus in excess of $10^{4.3}$ MPLD. At the age of 28 days, without having received any further treatment, the vaccinated mice were inoculated intracerebrally with 10 per cent virus suspension. Seven and one half and 15 hours after inoculation, pooled brains of groups of mice were titrated in normal animals. The data presented in Figure 4 and Table IV show that there is no alteration of HF virus growth in vaccinated mice as compared with the normal.

It can be argued that large HF inocula may institute an overwhelming infection, thereby obscuring possible relationships between the two viruses. To avoid the implications of this question, the above experiment was repeated using much smaller inocula.

In the experiment recorded in Figure 5, vaccinated mice received a total of two intraabdominal injections of vaccine lymph given 4 days and 2 hours before injection of HF virus. This simulates the condition of circulating vaccinia virus found in man following vaccination, and introduces into these experiments the possibility of interference between viruses. Animals prepared in this manner showed the same resistance to vaccinia L virus as those animals not receiving this injection ($10^{5.3}$ MPLD per ml). From the data (Figure 5 and Table V), there is a suggestion that vaccinial immunization has slowed

the multiplication of HF virus ($10^{5.1}$ MPLD inoculum). The two curves expressing the rates of viral multiplication tend to parallel each other. However, there is some suggestion that the curves may be converging. Repetition of this experiment with even smaller inocula of HF virus, and following the growth curve from an earlier time period, did not confirm this apparent difference. This is shown in Figure 6 with an HF inoculum of $10^{3.9}$ MPLD per ml. The crude data are given in Table VI.

In performing this last experiment (Figure 6), it was considered likely that some vaccinia virus would enter the brain following intraabdominal injection and be introduced, in turn, into normal mice when the brains were titrated. A control was therefore included to detect the possible further multiplication of this virus in normal mice. The control consisted of vaccinated animals (one booster injection five days before use) for a duplicate titration of pooled brains derived from vaccinated mice. Normal mice were used as before in titrating both normal and vaccinated groups. As indicated in Figure 6, the carry-over of free vaccinia virus does not appear to be a significant factor in this experiment.

Discussion

The results presented in this paper contribute to a clearer understanding of certain portions of the early phase of viral growth. In addition, the complete pattern of herpes virus in the mouse brain reveals a cycle of multiplication which conforms to and enlarges upon similar curves defined on the egg chorioallantoic membrane (75), and in tissue culture of rabbit corneal cells (74) and fibroblasts (81). This confirms the impression of Scott *et al.* (74) that the characteristic growth of herpes virus is independent of any peculiarity of the host tissues.

The initial rapid loss of recoverable activity during the first hour following the inoculation of HF virus, and the second slower decline, have been observed in mice as well as in tissue culture and chorioallantoic membrane. The first of these decreases is generally considered to be due to the rapid escape of up to 95 per cent of the injected material via the circulation (20). The factors underlying the second decrease are less clearly established. Schlesinger (71), in describing a similar picture for Western equine encephalomyelitis virus, has cautiously approached this second part of the growth curve, and has suggested two alternatives: (a) that the recoverable virus represents the development of a temporary equilibrium between the rate of disappearance of virus from the brain and an evolution of new virus; and (b) that it is comparable to the "constant

Discussion

periods" described for bacteriophage and influenza viruses. A more precise interpretation of the factors controlling this latent phase has been difficult where mice have been used as the growth medium. However, data obtained by use of the developing chorioallantoic membrane of the chick embryo indicate clearly that the concept of a constant period of virus growth is to be preferred. In the following discussion, the possible effects of circulating virus on the recoverable activity in the latent period are not considered to be important.

On the basis of this concept, the information presented in this paper, concerning the effect of removal of virus containing cellular debris from the growth curve inoculum, may be explained simply in terms of virus access and adsorption to brain tissue. Thus, if an essentially cell-free inoculum is introduced into the brain, the distribution of material is principally to the intraventricular spaces (34, 69), and from there to the circulation. The concentration of recoverable virus is found to drop rapidly during this process until, presumably, a point is reached where free virus has largely disappeared from the brain. What remains, perhaps 1 per cent (71) represents freshly adsorbed, infective material. The second decline then seen is more gradual, and usually proceeds to the point where virus is no longer recoverable. This phase is most easily thought of as one of viral penetration and/or the development of a state of non-infectivity (55). Now, if the inoculum should contain a large amount of potentially

infective cellular debris, it is most likely that escape of this material from the brain will be limited by purely mechanical factors. Tissue dissolution, however, will ordinarily occur, so that virus is eventually liberated. Thus, virus can be held at the principal site of adsorption over a relatively long period of time. The net effect of this process is that when brains injected with a sediment-containing fluid are examined during the latent, or constant, period, some virus is always recoverable; the amount depending primarily on the concentration of cellular debris in the inoculum. This explanation assumes that cellular debris contains virus either in an adsorbed or intracellular state. Henle (44) has shown that active influenza virus is found in allantoic membranes collected and ground late in the latent period. Similar findings have been reported for meningopneumonitis virus (77) and the T series of bacteriophages (26).

The indications are that cellular debris contains little infective material as compared to that found in the supernatant fluids. This fact alone may explain the small concentration of virus recovered directly after the inoculation of Sediment (Figure 2) into mice, and during the first six hours of the growth cycle. However, the possibility should also be considered that cellular debris may be partly removed by centrifugation when the brain pools are processed for titration, thus automatically reduced the infective titer of the material.

There are many factors present in the HF virus-mouse brain system which obscure the presence of the expected stepwise multiplication of virus. The principal one, as advanced by Scott *et al.* (75), is the continued adsorption of virus over a period of several hours, and its subsequent multiplication and liberation over a period of time. Although most of the virus can be expected to adsorb within about 15 minutes (75), those portions which do not adsorb rapidly obviously tend to obscure the development of any sudden "bursts" of virus activity. This, plus the presence of unreleased, active virus within the cell, should render such "bursts" even more unlikely. Nevertheless, if sufficient virus is released at the end of a growth cycle, the "bursts" will be pronounced enough to observe. Scott *et al.* (75) have estimated the increase in virus on the chorioallantoic membrane during the incremental period to be about 10-fold every two hours. This agrees with the findings recorded here in mice, and should provide a sufficient concentration of virus during a single cycle (100- to 1000-fold) to make these "bursts" apparent.

It is to be expected that a stepwise growth of virus would be most easily determined where all the susceptible cells were infected at one time. This has been consistently noted following inoculation with 10 per cent suspensions of virus. The single "burst" of activity at about 10 hours (Figure 2) lies just at the limits of normal variation ($\pm$ 2 S.E. = $\pm$ 50% of 1 log unit) and therefore may be of doubtful reliability.

However, the steps observed following inoculation with smaller quantities of virus have been analyzed and the majority of these appear to be significant. It is to be emphasized, nevertheless, that curves have been obtained with small inocula showing no evidences of stepwise increase. It is assumed that these curves are the result of overlapping growth cycles of virus.

A number of conditions have been established in mice which simulate the clinical use of vaccination in the treatment of herpes simplex. These have included (a) no immunity, (b) vaccinial immunity with probable circulating antibody, but little or no circulating virus, and (c) immunity and large concentrations of circulating virus. Inasmuch as the question of a herpes-vaccinia relationship entails the possibility of viral interference, these conditions may be expected to fulfill most of the requirements for interference as set forth by Henle (45). The failure of any observable alteration to occur in the growth pattern of HF virus suggests more forceably that herpes simplex virus and vaccinia virus are unrelated. However, it is wise to approach this apparently logical conclusion with some caution, since the localized, intracellular, and generally benign nature of herpes simplex virus in patients cannot be duplicated in experimental animals. This implies that perhaps the precarious balance between latent and activated herpes simplex virus in patients is the key to a possible herpes-vaccinia relation, and that the effect of vaccinia virus,

or its antibody, is directed at blocking the activation of small quantities of herpes virus, rather than preventing the establishment of a new infection in a previously unaffected area. In addition to subtle factors of cellular immunity which are not adequately understood, this hypothesis may then involve a question of concentration of virus. In other words, only small amounts of herpes virus may be required to re-establish a local lesion in man, whereas much larger amounts may be needed to initiate infection in mice by the intracerebral route of inoculation. In a like manner, much smaller amounts of vaccinia virus would then be required to suppress the former type of lesion than to suppress the latter. The unknown factor of the penetrability of brain tissue by vaccinia virus therefore becomes an important consideration in the experiments just discussed.

A conflicting note in the above reasoning is provided by two reports that vaccinia virus can activate latent herpetic infections in rabbits (52, 66). Perdrau (63), among others, was unable to confirm this. Sharlit (76) has cited a case where handling of smallpox vaccine was followed by a recurrence of herpes. Although there is considerable question as to the value of these reports of herpes activation by vaccinia, they point nevertheless to other mechanisms that may function to suppress the recurrent lesions. Thus, it is possible that vaccinia virus could function as a continuous, specific or

[illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible]

[illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible] [illegible]

non-specific, activator of herpes virus, thereby raising the level of herpes antibody locally, and so preventing the development of symptoms.

Summary

1. Mouse-adapted strains of herpes simplex virus failed to agglutinate red cells of two different animal types when tested by direct and indirect methods. Agglutination did not occur with tannic acid treated cells or with a preparation of liver cell nuclei.

2. Growth curves were determined for three different concentrations of herpes simplex virus in the brain of the mouse. The patterns obtained agree with curves defined in tissue culture and on the chorioallantoic membrane. This confirms the impression of Scott *et al.* that the characteristic growth of herpes simplex virus is independent of the cell type.

3. The presence of cellular debris in the viral inoculum results in the recovery of significant quantities of virus during the latent period of virus growth. Removal of this debris by increasing the speed of centrifugation to 3000 rpm for from 45 to 60 minutes minimizes this effect. The probable mechanism is discussed.

4. The development of herpes virus in mice immunized against vaccinia was not significantly different from that in normal animals. The significance of this finding is discussed in the light of present concepts of vaccinial therapy of recurrent herpes infection.

TABLE I

The Effect of Time and Speed of Centrifugation on Virus Growth*

Inoculum	Hrs. after inoculation	Survival rate in mice inoculated with Dilution of virus mixture					Titer of virus expressed as MPLD per ml	Negative log of LD_{50} per 0.03 ml
		$10^{-0.5}$	10^{-1}	10^{-2}	10^{-3}	10^{-4}		
Supernate I	0			0/5	1/5	5/5**	43×10^3	3.4
	3½		2/5	4/5	5/5		360	1.3
Supernate II	0		0/5	1/5	4/5	4/5	69×10^2	2.7
	3½		4/5	5/5	5/5		66	0.4
	6	3/5	5/5	5/5			33	0.3
	9			0/5	2/5	5/5	26×10^3	3.2
Sediment	0		0/5	3/5	5/5		16×10^2	1.8
	3½		1/5	4/5	5/5		560	1.5
	6	1/5	3/5	5/5			130	0.9
	9			0/5	2/5	5/5	26×10^3	3.2

* The data in Figure 1 are taken from this table.

** Fractions represent number of mice surviving in numerator, number inoculated in the denominator.

TABLE II

Growth Curve of Virus in the Brains of Mice Inoculated with a 10^{-1} Suspension (19 x 10^9 MPLD) of HF Virus*

Hours after inoculation	Survival rate in mice inoculated with Dilution of virus mixture										Titer of virus expressed as MPLD per ml	Negative log of LD_{50} per 0.03 ml
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
0		0/5	4/5	4/5	5/5						15 x 10^3	2.7
1	0/5	2/5	4/5	5/5							36 x 10^2	2.3
1½	0/5	4/5	5/5								11 x 10^2	1.6
2	1/5	4/5	4/5	5/5							690	1.6
3	1/5	4/5	5/5								560	1.5
4	3/5	4/5	5/5								220	1.0
5	1/5	5/5	4/5	5/5							560	1.5
6		2/5	5/5	5/5							25 x 10^2	2.2
7½		0/5	3/5	5/5							16 x 10^3	2.8
9		0/5	1/5	4/5	5/5						56 x 10^3	3.5
10½		0/5	1/5	4/5	5/5						56 x 10^4	4.5
12				2/5	3/5	4/5	5/5				56 x 10^4	4.7
15					0/5	2/5	2/5	4/5	4/5	5/5	82 x 10^6	7.2

For explanation of figures see Table I.

* The inoculum, and the brain pools used to determine the points, were centrifuged at 2000 rpm for 30 minutes. The data in Figure 2 are taken from this table and from the following table.

TABLE III

Growth Curves of Virus in the Brains of Mice Inoculated with Three Different Dilutions of HF Virus *

Size of inoculum	Hrs. after inoculation	Survival rate in mice inoculated with Dilution of virus mixture								Titer of virus expressed as MPLD per ml	Negative log of LD_{50} per 0.3 ml
		$10^{-0.5}$	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
	0			0/5	4/5	5/5				11×10^3	2.6
	1	1/5	4/5							130**	0.8
	2	2/5	3/5							100**	0.8
	4	4/5	5/5							33**	0.2
10^{-1}	6	2/5	5/5	4/5						100	0.7
	7½		0/5	3/5	5/5					16×10^2	1.8
66×10^6	9			1/5	3/5	5/5				73×10^2	2.7
MPLD	10½			0/5	1/5	4/5	4/5			73×10^3	3.6
	12				0/5	3/5	5/5			16×10^4	3.8
	15					0/5	1/5	4/5	5/5	56×10^5	5.5
	18						0/5	3/5	5/5	16×10^6	5.8
	1	3/5	5/5							33**	0.3
	6	5/5	5/5							0	0
	7½		4/5	5/5						66**	0.4
10^{-3}	9		0/5	2/5	4/5					36×10^2	2.3
	10½		0/5	2/5	5/5					26×10^2	2.1
26×10^5	12			0/5	3/5	5/5				16×10^3	2.8
MPLD	15			0/5	1/5	2/5	5/5			92×10^3	4.0
	18				0/5	3/5	3/5	5/5		31×10^4	4.2
	24						0/5	0/5	5/5	76×10^5	6.5
	9	5/5								0	0
	10½	5/5	5/5							0	0
	12	4/5	5/5							33**	0.2
	14		4/5	5/5						66**	0.4
	16		1/5	4/5	5/5					560	1.5
10^{-5}	18		0/5	2/5	3/5					46×10^2	2.5
	20		0/5	1/5	2/5	5/5				92×10^2	3.0
23×10^3	22			0/5	0/5	5/5				80×10^3	3.5
MPLD	24			0/5	1/5	3/5	5/5			73×10^3	3.7
	26			0/5	2/5	5/5				26×10^3	3.1

TABLE III continued

28		0/5	2/5	5/5			26×10^4	4.0
30		0/5	2/5	3/5	5/5		46×10^4	4.5
32			0/5	4/5	5/5		11×10^5	4.6
35		0/5	2/5	4/5	5/5		36×10^4	4.3
38			0/5	1/5	5/5		43×10^5	5.4
42				0/5	3/5	4/5	22×10^6	6.0
48				0/5	4/5	5/5	11×10^6	5.6

For explanation of figures see Table I.

* The data in Figure 3 are taken from this table. The inoculum, and the brain pools used to determine the points, were centrifuged at 3000 rpm for from 45 to 60 minutes.

** Value based on deaths occurring in only two virus dilutions.

TABLE IV

Growth Curve of Virus in Mice Immunized Against Vaccinia

Virus grown in	Hours after inoculation*	Survival rate in mice inoculated with Dilution of virus mixture 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Titer of virus expressed as MPLD per ml	Negative log of LD_{50} per 0.03 ml
Normal mice (control)	$7\frac{1}{2}$	0/5	2/5	3/5	5/5				46×10^2	2.5
	15					0/5	4/5	4/5	15×10^6	5.7
Vaccinated mice	$7\frac{1}{2}$		0/5	3/5	5/5				16×10^3	2.8
	15					0/5	3/5	5/5	16×10^6	5.8

* 10^{-1} inoculum, MPLD not calculated. The data in Figure 4 are taken from this table.

TABLE V

Growth Curve of Virus in Mice Immunized Against Vaccinia

Virus grown in	Hours after inoculation*	Survival rate in mice inoculated with Dilution of virus mixture 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Titer of virus expressed as MPLD per ml	Negative log of LD_{50} per 0.03 ml
Normal mice (control)	17	0/5	1/5	2/5					92×10^2 or >	3.0 or >
	20			0/5	4/5				12×10^3	3.6
	23			0/5	1/5	3/5	5/5		73×10^4	4.7
	36				0/5	2/5	4/5		36×10^5	5.3
	42					0/5	1/5	4/5	56×10^6	6.5
Vaccinated mice	17	0/5	3/5	4/5					860	2.0
	20		0/5	2/5	5/5				26×10^2	3.1
	23			0/5	2/5	4/5	5/5		73×10^3	4.3
	36				0/5	3/5	4/5		23×10^5	5.0
	42					0/5	2/5	5/5	26×10^6	6.1

For explanation of figures see Table I

* 10^{-4} inoculum, 12×10^4 MPLD. The data in Figure 5 are taken from this table.

TABLE VI

Growth Curve of Virus in Mice Immunized Against Vaccinia

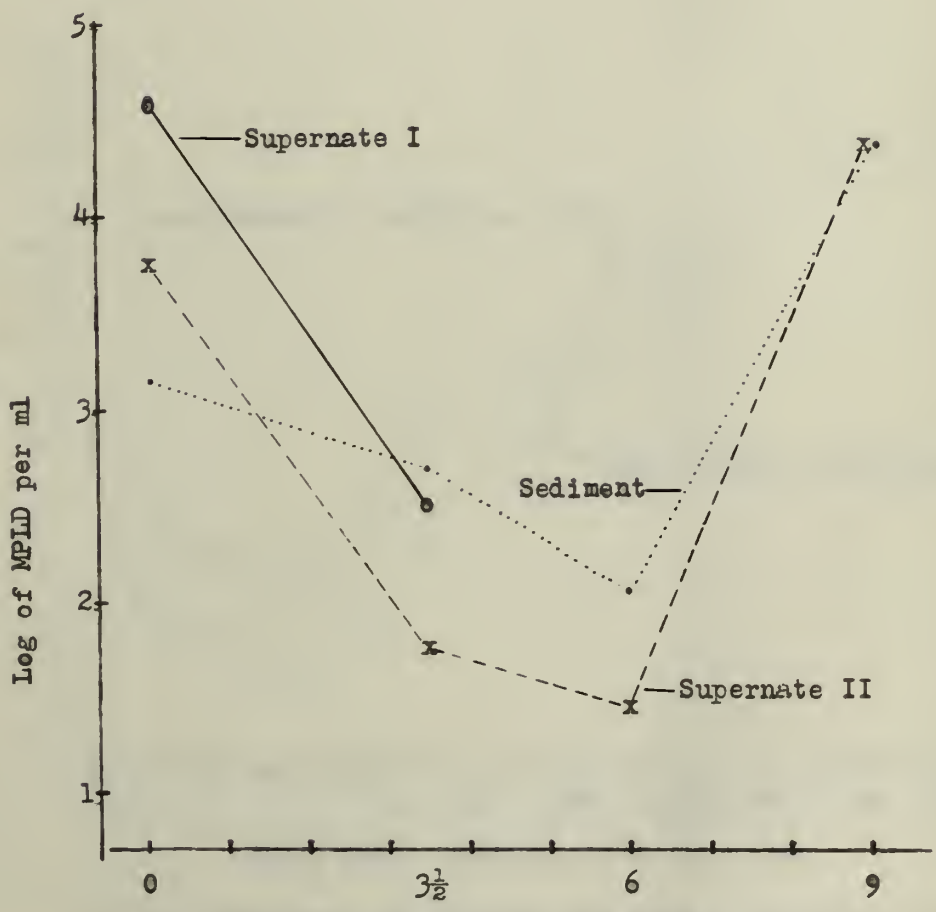
Virus grown in	Hours after inoculation*	Survival rate in mice inoculated with Dilution of virus mixture							Titer of virus expressed as	
		$10^{-0.5}$	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	MPLD per ml	Negative log of LD_{50} per 0.03 ml
Normal mice (control)	12	5/5	5/5	4/5	4/5				33	0.4
	14	4/5	5/5	5/5					33	0.2
	17	0/5	1/5	5/5					430	1.4
	20			0/5	4/5	4/5	5/5		15×10^3	2.7
	23				0/5	2/5	4/5	5/5	36×10^4	4.3
	36					0/5	4/5	5/5	11×10^5	4.6
Vaccinated mice	12	5/5							0	0
	14	4/5	5/5						33	0.2
	17	1/5	0/5	1/5	5/5				600	2.3
	20			0/5	3/5	5/5			16×10^3	2.8
	23				0/5	5/5			76×10^3	3.5
	36					0/5	3/5	5/5	16×10^5	4.8
Vaccinated mice and titrated in vaccinia immune animals	17			1/5	5/5				43×10^2	2.4
	20			1/5	5/5				43×10^2	2.4
	23				0/5	4/5	5/5		11×10^4	3.6

For explanation of figures see Table I.

* 10^{-5} inoculum, 76×10^2 MPLD. The data in Figure 6 are taken from this table.

Figure 1

The effect of time and speed of centrifugation on virus growth (early phase)



Supernate I: prepared by centrifuging inoculum at 2000 rpm for 30 minutes.

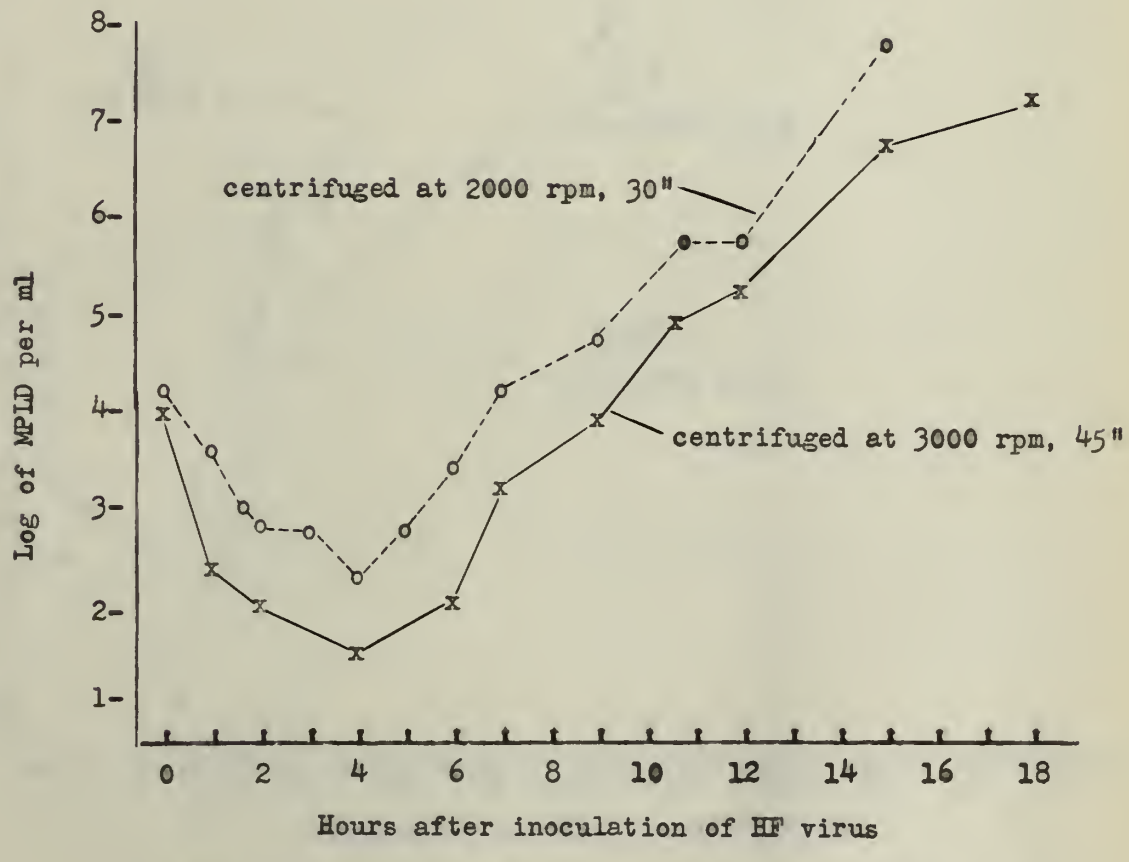
Supernate II: prepared from Supernate I by recentrifuging at 3000 rpm for 45 minutes.

Sediment: resuspended sediment from Supernate II inoculum.

The brain pools used to determine the points were centrifuged at the same speed - 2000 rpm for 30 minutes

Figure 2

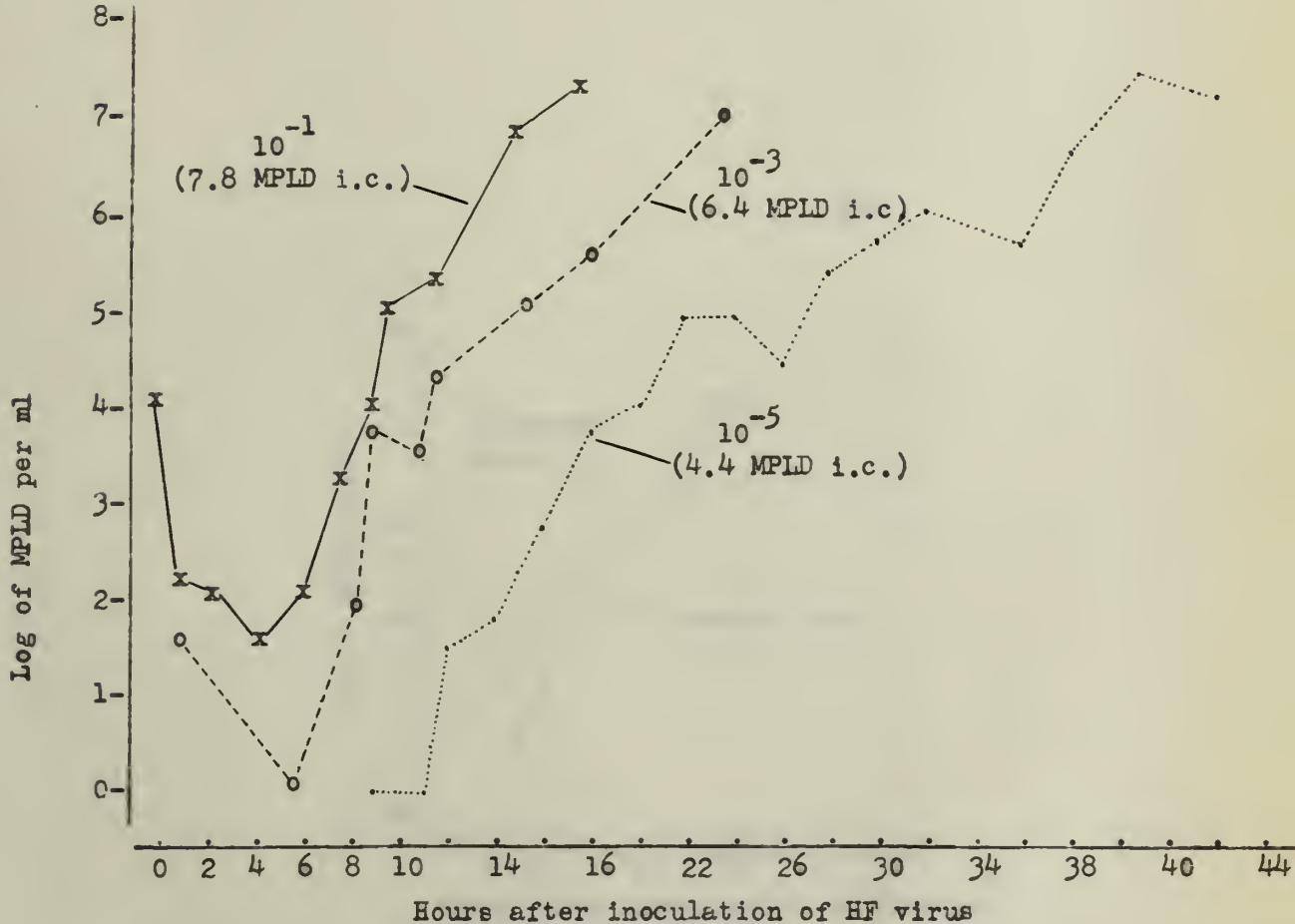
Growth curves of virus in the brains of mice inoculated with a 10^{-1} suspension of HF virus
The effect of time and speed of centrifugation on virus growth*



* The inocula and the brain pools used to determine the points were centrifuged at the speeds noted.

Figure 3

Growth curves of virus in the brains of mice inoculated with three different concentrations of HF virus



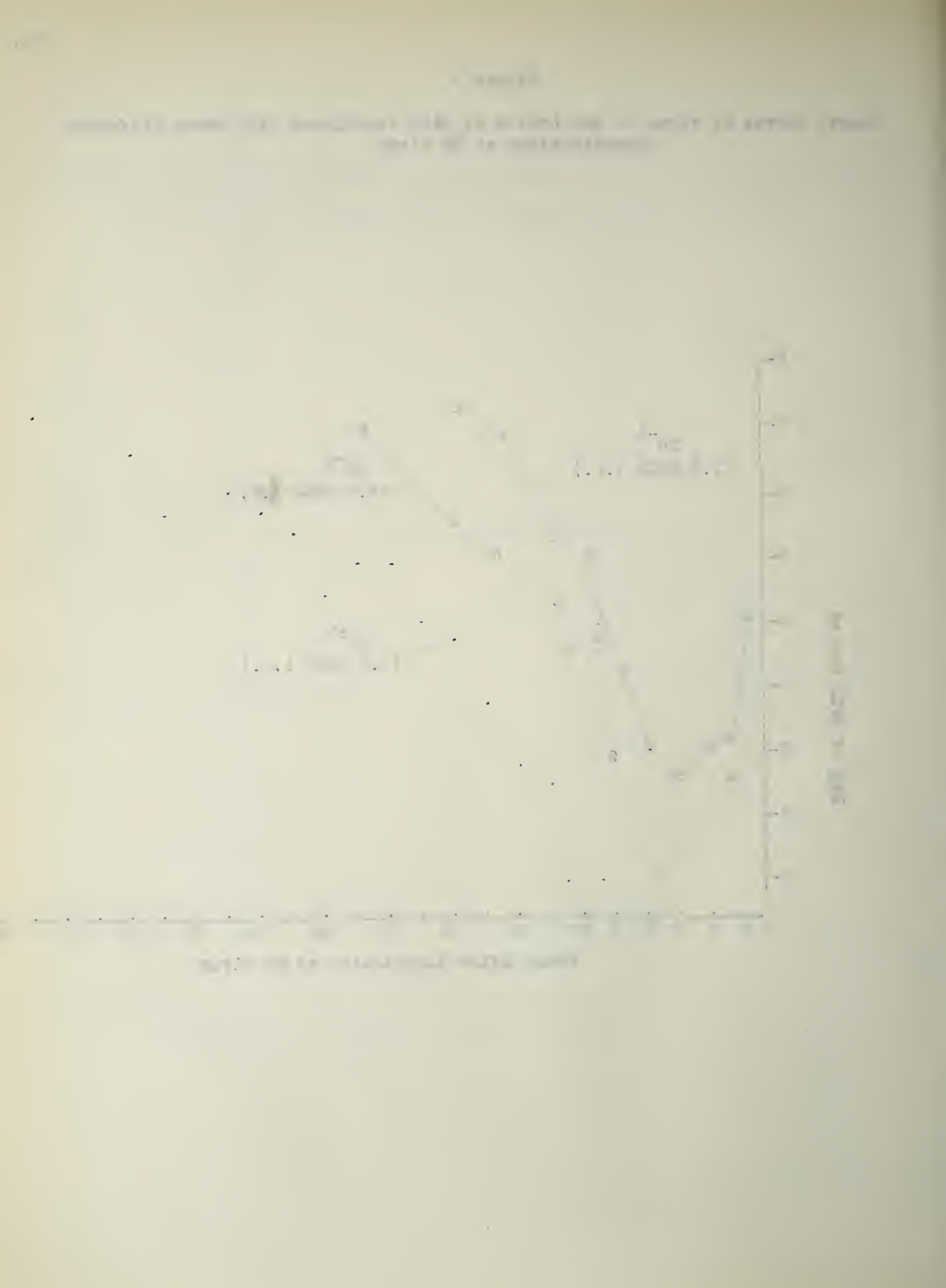
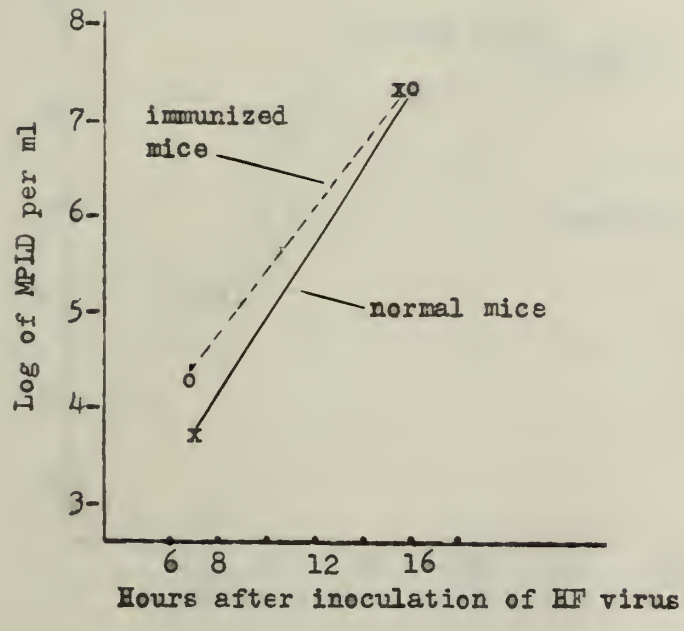


Figure 4

Growth curve of virus in mice immunized against vaccinia
10^{-1} inoculum ($10^{4.3}$ MPLD per ml)



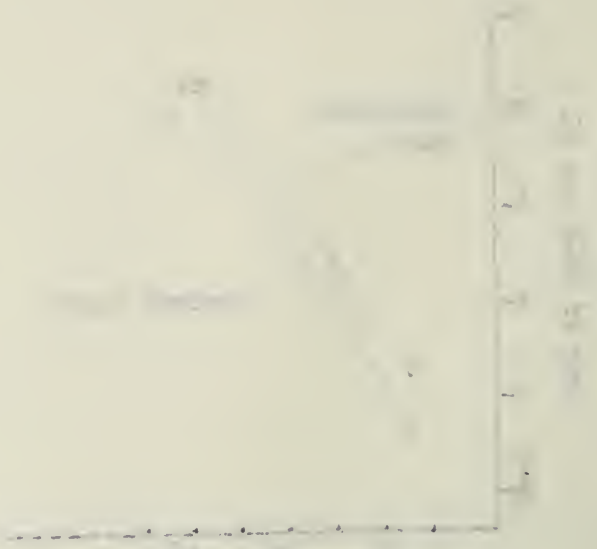
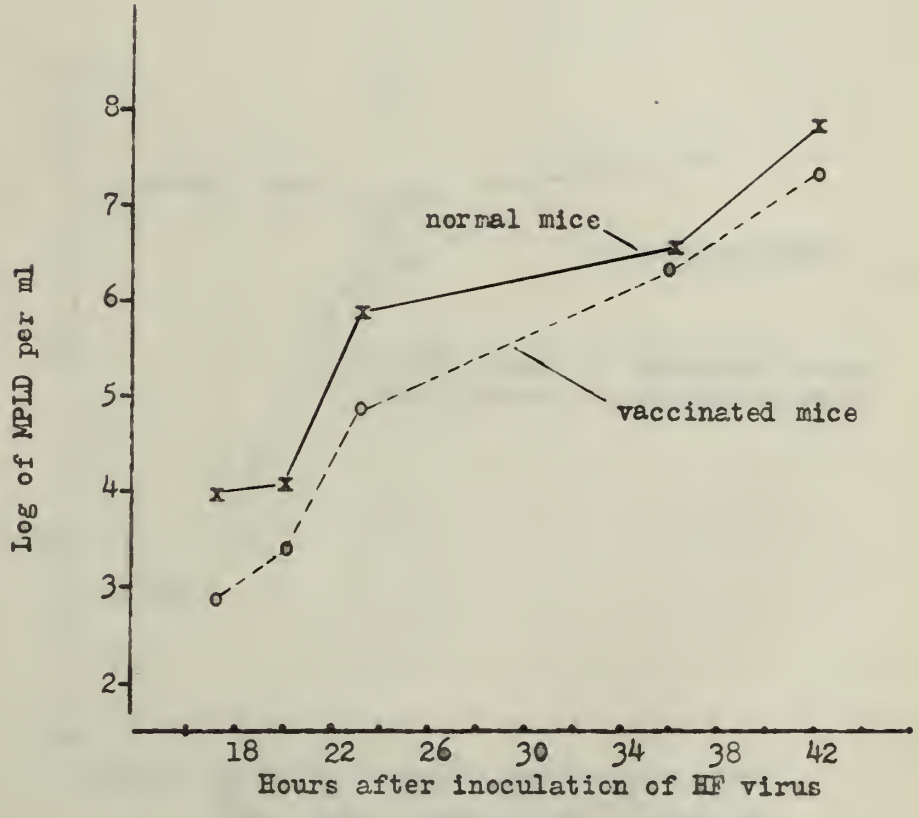


Figure 5

Growth curve of virus in mice immunized against vaccinia 10^{-4} inoculum ($10^{5.1}$ MPLD per ml)



Vaccinated mice received two intraabdominal injections of vaccine lymph before use

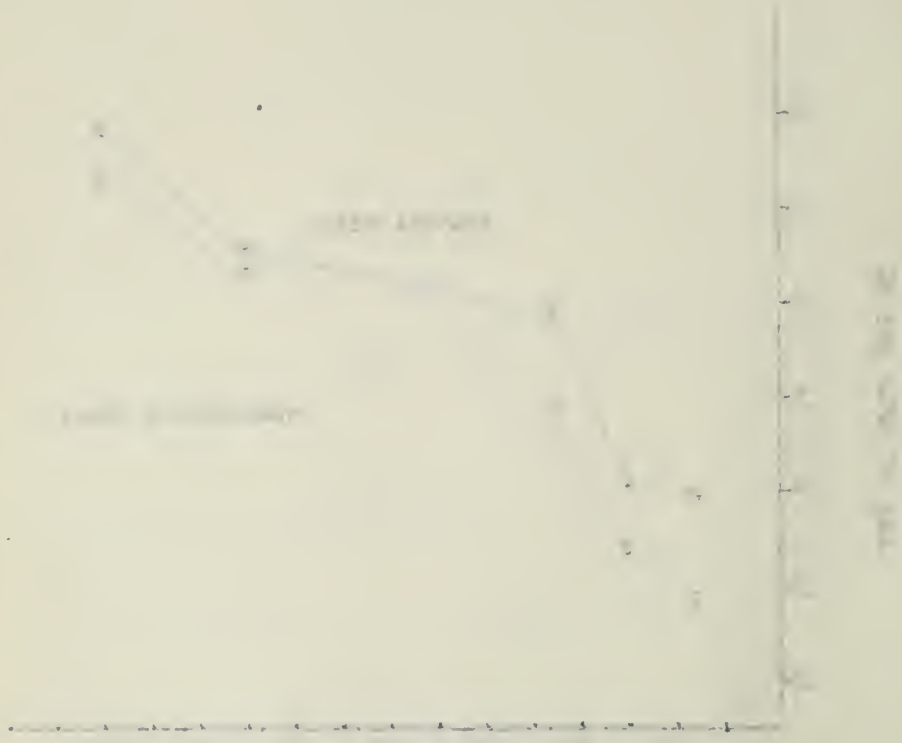
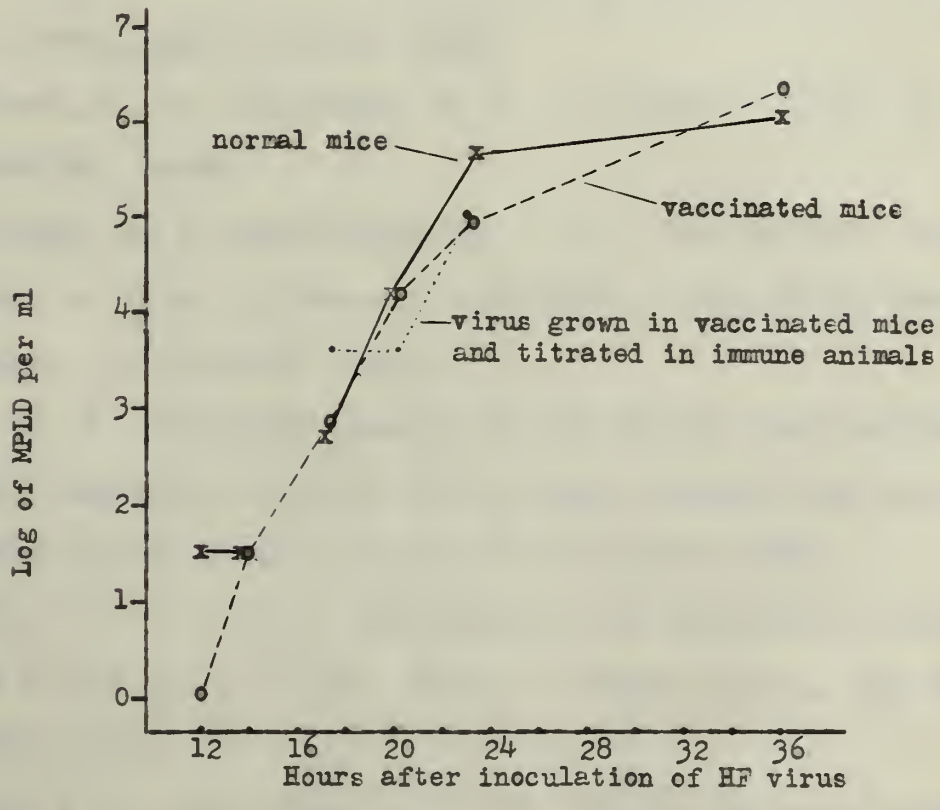


Figure 6

Growth curve of virus in mice immunized against vaccinia 10^{-5} inoculum ($10^{3.9}$ MPLD per ml)



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