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Development and Characterization of a Model Latent Herpes Simplex Virus Infection in Cultured Cells

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DEVELOPMENT AND CHARACTERIZATION OF A MODEL LATENT HERPES SIMPLEX VIRUS INFECTION IN CULTURED CELLS

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ABSTRACT

Herpes simplex virus is the classic example of a virus which persists indefinitely in the host following the initial infection. Recurrent episodes of active virus growth characterize the infection in some individuals. In addition to the periodic flair-ups of active virus growth, the persistence of virus may be expressed in a more subtle way. It is possible that one or more types of human cancer result from the persistence of this virus in the host.

The exact mechanism by which the virus is maintained in the host between outbreaks of active growth is not known. It is usually not possible to isolate the virus between the outbreaks and the virus is said to be latent during this period. Most of the available evidence indicates that the virus exists in a nonreplicating form when it is latent.

This dissertation describes the establishment and characterization of a model latent herpes simplex virus infection in cell cultures. Cultures of rabbit kidney cells were infected with approximately one infectious unit of virus per 100 cells and incubated at 41C for 1-7 days. No evidence of virus growth was seen at 41C and infectious virus disappeared from the cells very rapidly at this temperature. Two-hundred infected cultures were transferred to 37C after incubation at 41C. In 43% of these cultures virus was recovered within 3 days after incubation at 37C. In 16% of these cultures no virus was recovered after incubation at 37C for

up to 150 days. In the remaining 41% of the cultures, however, virus growth occurred but only after lag periods of variable length. The longest lag period was 45 days and the average lag period was 15.3 days. During the period when no evidence of virus growth was seen, treatment of the cells by freeze-thawing or by sonication failed to yield infectious virus. Once active virus growth occurred, virus was able to be isolated from the cultures and this virus was neutralized by commercial herpes simplex virus antiserum.

Quantitative studies indicated that 0.25-1% of the inoculum virus was able to survive incubation at 41C for up to 6 days and replicate after transfer to 37C. Immunofluorescence studies indicated that virus-specific antigens were produced at 41C. Fluorescence microscopy of cells stained with acridine orange indicated that the block in virus replication occurred early in the infection cycle. Characteristic features of cells infected with virus at 37C were absent from the cells infected at 41C.

Although virus failed to replicate in rabbit kidney cells at 41C, control cells preincubated at 41C were fully competent to replicate exogenous virus immediately after transfer to 37C.

An attempt was made to establish the latent infection in cells directly at 37C by using a very small inoculum of virus. The latent infection could not be established in this way. When virus was added directly onto cells at 37C, active virus growth was always seen within 3 days or no growth occurred at all in cultures maintained for up to 30 days.

A latent infection similar to that established in rabbit kidney cells was established in Wistar-38 cells. However, attempts to establish a similar infection in human kidney cells were unsuccessful. The basis for this cell-dependent difference was not investigated.

Cultures were treated with certain hormones and chemical agents in attempts to modify the latent infection. Hormones that were used include hydrocortisone, 17 B-estradiol, progesterone, L-thyroxin and L-epinephrine. Treatment of cultures with progesterone at the time that the cultures were infected with virus and incubated at 41C resulted in a significant reduction in the number of cultures from which virus was recovered after transfer to 37C. The other hormones did not affect the latent infection in this way.

Three chemical agents, 5-bromo-2-deoxyuridine, 5-iodo-2-deoxyuridine, and puromycin increased the reactivation rates of virus from cultures when the cultures were treated with the agent at the time of infection with virus and incubation at 41C. When cultures were treated with 5-bromo-2-deoxyuridine at the time of transfer from 41C to 37C (after the latent infection had already been established) the overall recovery rate was the same from treated cultures as from control cultures. However, the average lag period of the treated cultures was significantly increased.

Finally, treatment of cultures with neutral red dye and exposure of these cultures to strong visible light had no effect on the overall recovery rate but did increase the average lag period of the treated cultures.

INTRODUCTION

It is becoming clearer each day that many viruses, if not all viruses, have the ability to persist indefinitely in the host following acute or subacute primary infections. While it may be that most of these persistent infections cause the host no ill effects, it is likewise apparent that in predisposed individuals serious consequences may result from the long-term persistence of virus. Such conditions as subacute sclerosing pan encephalitis, progressive multifocal leukoencephalopathy, and perhaps even multiple sclerosis as well as a number of other degenerative diseases of the central nervous system have been definitely proven to be or are suspected of being sequelae of viral infections. In addition to these rare degenerative diseases, a variety of neoplastic diseases in animals are known to be the long-term result of persistent viral infections. Certain malignant conditions in man are also suspected of being the result of previous viral infections. As more and more of the acute, febrile infections become controlled by one method or another, the persistent viral infections and the consequences of these long-term infections come to dominate the thinking of virologists.

Herpes simplex virus (HSV) and other members of the herpesvirus group (varicella-zoster virus, cytomegalovirus and the Epstein-Barr virus) are classic examples of viruses which persist indefinitely in the

host following primary infections. Although it has been known for a long time that herpes simplex virus persists in the host, we still do not completely understand the relationship of the host and the virus that accounts for this persistence. The virus is described as being latent because no direct evidence of virus is normally seen between outbreaks of active virus growth.

A number of in vivo and in vitro model systems have been developed in an attempt to determine what is happening during the latent infection. In vivo model systems have been developed which seem to approximate the natural latent infection very closely. However, we do not fully understand what is happening in these model systems. In vitro cell culture systems have also been established. The cell culture model systems are much simpler than the in vivo models in that they involve interactions between the virus and the host at the cellular level only. Carrier culture infections are one type of in vitro model latent infection which has been described. Virus multiplication is not blocked in this type of infection but occurs at a very low rate. At least one model infection has been described in which virus growth apparently is completely blocked.

We have established a model latent HSV infection that has certain advantages over other in vitro models. It is the purpose of this paper to describe the establishment of this latent infection and to characterize certain features of it. Originally the infection was established in rabbit kidney cells. The infection in this cell type has been most thoroughly

investigated. In addition to characterizing the infection in rabbit kidney cells, attempts were made to duplicate the infection in two cell types of human origin. Finally, attempts were made to modify the latent infection by treating the cells with various chemical agents which we had reason to believe would affect the latent infection.

HISTORY

HSV is a highly infectious agent. Approximately 90% of the population have antibodies to this virus by the age of 15 years (1). Initial contact with the virus usually occurs at an early age, but most primary infections are subclinical. When symptoms of infection do occur, a characteristic feature of the disease is the formation of vesicular eruptions localized in various areas of the body. Although any area of the skin is susceptible to infection, the virus shows preference for mucous membranes (2-5). The most frequent manifestation of primary herpetic infection is acute gingivostomatitis, characterized by vesicular lesions covering the tongue and mucous membranes of the mouth and oral cavity.

Infection of the mucous membranes of the genital tract may occur in either sex. Acute vulvovaginitis as a manifestation of HSV infection was first established by Slavin and Gavett in 1946 (6). Since that time it has become apparent that herpetic infection of the female genital tract is the most common viral gynecological disease (7).

Primary genital infection of women during pregnancy may result in transmission of the virus to the infant at birth. In the neonate, the virus may become systemic and produce a generalized, often fatal, infection (8).

Keratoconjunctivitis is a much less common herpetic infection but is potentially serious because the vesicular eruptions may ulcerate and lead to scarification of the cornea and blindness (5).

Systemic involvement may accompany any of these syndromes of primary HSV infection. Frequently there is an enlargement of regional lymph nodes and in some cases a viremia is present. Temperatures as high as 105° F may be seen. HSV has neurotropic properties and in certain cases, systemic involvement leads to a meningitis or encephalitis (5).

Strains of HSV have been divided into 2 distinct subgroups based on biological and antigenic differences (9-17). The two subgroups are referred to as herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2). More than 90% of the genital infections caused by HSV are due to HSV-2 while most non-genital infections are with HSV-1 (9).

A well-known feature of HSV is its ability to establish a latent infection in the host following the primary infection. The common "cold sore" or "fever blister" is the classic example of recurrent HSV infection resulting from latency following a primary case of gingivostomatitis. Recurrent cervicitis and vulvovaginitis frequently follow primary genital infection of the female with HSV-2 (18). Although the primary infections are usually more severe and longer lasting, recurrent infections are characterized by the same localized outbreak of vesicular lesions as the primary infection.

The nature of the host-virus relationship that accounts for the latent infection is not fully understood. A widely-held view is that the virus is associated with nerve tissue between the recurrent outbreaks. Cushing was perhaps the first to suspect the involvement of nerve tissue (19). He noticed that in two cases of surgical removal of the gasserian ganglion for neuralgia, herpetic lesions occurred on the sixth and seventh postoperative day.

This early observation was substantiated by Carton and Kilbourne (20, 21). They reported that surgical section of the posterior root of the trigeminal nerve for the treatment of trigeminal neuralgia resulted in the precipitation of recurrent herpetic lesions in 44 out of 47 patients. Lesions appeared between 48-96 hours after surgery and in all cases the lesions first appeared in areas of the skin that were innervated by the sectioned nerve. HSV was recovered from the vesicular fluid from the lesions by injection into mice. However, virus was not recovered from mice that were injected with nerve tissue removed at surgery. It was postulated that the virus resided in surface epithelial cells and that sectioning of the nerve activated virus growth.

In at least 2 other studies (22, 23) attempts have been made to demonstrate the virus in ganglion cells at the time of nerve section. Suspensions of nerve tissue were prepared from 7 different patients and inoculated intracranially into rabbits. None of the animals showed signs of infection and virus was not recovered from any animal. However, 5 of the 7 patients developed typical herpetic lesions following surgery.

Although it has not been possible to isolate the virus from ganglion tissue at the time of removal of the tissue from the body, this does not constitute proof that the virus resides outside the ganglion tissue and that sectioning of the nerve root merely provides the stimulus needed for virus activation. Recently, Stevens and Cook (24, 25) have developed experimental models using mice and rabbits and their studies indicated that virus does reside in the ganglion tissue. Infection of mice was initiated by inoculation of HSV into the rear foot pads. Following this treatment about 30% of the animals died of encephalitis and 15% of the animals showed no symptoms of infection. The remaining animals developed hind limb paralysis to varying degrees and about half of the animals in this group recovered. The spinal ganglia that innervated the inoculated site were removed from animals on each day after infection and assayed for virus. Virus was first recovered from ganglion tissue 2 days after infection. Peak virus titers were obtained at about day 4 and virus disappeared by the seventh or eighth day. The presence of viral antigens closely paralleled the presence of infectious virus. Virus antigens did not remain after the disappearance of infectious virus. After mice recovered from paralysis, virus could not be detected in ganglion cells of these animals at the time that the tissue was removed from the animals. However, after explantation of the ganglion cells in vitro, infectious virus appeared in the supernatant fluids within 5 days. The virus recovered in this manner was indistinguishable from the inoculum virus as indicated by neutralization tests. This study indicated that the

ganglion cells contained latent virus which was released after cultivation of the cells *in vitro*.

In this same study rabbits were infected by dropping a suspension of the virus onto the surface of their eyes. An acute infection of the eyes occurred following this procedure and virus could be recovered from the trigeminal ganglia 5 days after infection. By the ninth day virus had disappeared from the surfaces of the infected eyes and from the ganglion cells. During the next 4-8 months virus intermittently appeared in the tear films from the infected eyes. At a point when no virus was recovered from the tear film of a rabbit for at least 1 month, the ganglion cells were removed from the animal and placed in culture. No virus was detected in the ganglion cells at the time of explantation but infectious virus did appear in the supernatant fluids from 7 of the 12 ganglia that were cultured. In 4 ganglia cultures virus was recovered during the first week of cultivation. From 2 ganglia virus was recovered within 2 weeks and in 1 culture virus was recovered after 3 weeks. The authors concluded from the studies with both mice and rabbits that the virus was able to establish a latent infection in sensory ganglia and that these experiments directly support the concept that sensory ganglia are the reservoir of the virus during the latent infection.

Two hypotheses have been proposed to explain the nature of the latent infection; the "dynamic state" hypothesis and the "static state" hypothesis (26). According to the "dynamic state" hypothesis, the latent infection is maintained by continued multiplication of virus in a

small number of cells. In the "static state" hypothesis, the virus is maintained in certain cells in a non-replicating form. According to this hypothesis, various stimuli are able to cause resumption of active viral replication. Stevens and Cook favor the "static state" hypothesis because virus could not be detected in the ganglion cells at the time of their removal from the animal although virus could be detected soon after the ganglion cells were cultured in vitro. However, as long as it is not possible to determine the minimum amount of infectious virus that is needed before detection is possible, it cannot be stated for certain that a small amount of replicating virus was not present.

The evidence that HSV resides in cells of the sensory ganglia, presumably in a non-replicating form, between outbreaks of recurrent infection is not conclusive. Certain studies have indicated that the latent infection may be maintained by active multiplication of virus in a small number of cells. Kaufman, Brown and Ellison (27) infected 15 rabbits with HSV by corneal scarification. After the initial lesions healed, daily cultures of the tear film were obtained and inoculated onto human amnion cells. During the study, virus was isolated 73 times while only 16 recurrent corneal ulcers were observed.

In the same study, corneal tear films and saliva specimens were obtained from 35 individuals, 11 of whom had no history of recurrent herpetic infection. Four of the individuals who had no history of recurrent infection had episodes of virus shedding during the period of study. Among the individuals who did have a history of recurrent herpetic

infection, at least 3 people had 1 or more episodes of virus shedding. In neither group did any individual develop any herpetic lesions during the study. The authors postulated that virus production may occur continually at a low level and that clinical infection is a result of change in resistance to the virus by tissues normally bathed in virus.

In another study (28) a group of 418 healthy adult males were infected intranasally with one of five types of respiratory viruses. Nasal cultures, throat swabs, saliva specimens and conjunctival swabs were taken every other day and cultured in Wistar-38 cells and human embryonic kidney cells. During the course of this study, HSV (not one of the viruses which was used to infect the men originally) was isolated 27 times from 21 of the volunteers. One subject had 3 positive cultures, four subjects had 2 and sixteen subjects had 1 positive culture. Virus was most often cultured from saliva, throat swabs and nasal cultures. There was no direct relationship between isolation of the virus and subsequent development of herpetic lesions, although 4 cases of recurrent infection did develop after virus isolation. Because each of the 21 individuals had significant levels of neutralizing antibody in his serum, it is unlikely that the virus represented primary infections. These data are consistent with the concept of chronic virus multiplication with intermittent shedding of virus. Since virus was recovered from eyes, nose, mouth and throat (though not from all sources in any one individual) it is possible that the virus resides in glands that secrete into these areas rather than in sensory ganglia.

In the 4 cases where herpetic lesions occurred, the outbreaks followed cases of respiratory illness. It is possible that the illness did not activate a nonreplicating virus but rather altered the sensitivity of tissues to the virus making them more susceptible to infection with virus that was already present in an active form.

In a following study (29) saliva cultures were taken three times per week from 10 healthy volunteers who had high titers of neutralizing antibody to HSV. Over a 5 month period 607 samples were taken. Of these, 22 were positive for HSV. Of the 22 positive specimens, 11 occurred within 7 days of the onset of herpetic lesions and 11 in the absence of lesions. This confirmed earlier reports that the virus could be isolated in the absence of demonstrable pathology, and also indicated that if serial specimens were taken, the rate of virus recovery in the absence of lesions could be much higher than the earlier reported 5%.

Further evidence that virus multiplication may occur slowly between outbreaks of recurrent disease were reported by Dawson, Togni, Moore and Coleman (30). They removed cornea and corneal stroma tissue from five patients who suffered from recurrent herpetic keratoconjunctivitis and observed the tissues by electron microscopy. Although minced tissue and corneal tear film specimens failed to yield virus when they were cultivated, virus particles indistinguishable from HSV were observed by electron microscopy. These results are open to several interpretations. It is possible that chronic multiplication of virus does occur at the site of recurrent infections between outbreaks of the disease.

However, it is also possible that the virus particles observed by electron microscopy were noninfectious and totally unrelated to maintenance of the latent infection.

Up to this point two possibilities have been considered: that virus resides in sensory ganglion cells between outbreaks of infection (presumably in a nonreplicating form) or that virus resides in some other tissue (presumably being maintained by a low rate of continued multiplication). A third possibility is that virus resides in a nonreplicating form in cells other than sensory ganglion cells. HSV-2 has been implicated in the etiology of cervical carcinoma. Initially the implication was based on epidemiologic evidence (31, 32) but since then other evidence in support of this possibility has been obtained (33-36). If HSV-2 is etiologically related to cervical carcinoma, it would indicate that the virus can exist in a nonreplicating form in these epithelial cells.

Direct evidence that HSV-2 may occur in epithelial cells in a nonreplicating form has been obtained (35,36). Exfoliated cervical epithelial cells were obtained from patients with histologically proven cases of preinvasive or invasive cervical carcinoma. Smears made from all patients showed both the normal epithelial cells and abnormal dyskaryotic cells. After being stained, the normal cells were readily distinguishable from the abnormal cells. These cells were then treated with rabbit antiserum that had been made against HSV-2-infected hep-2 cells and conjugated to fluoresceinisothiocyanate. An average of 21% of the dyskaryotic cells in smears made from patients with preinvasive

cervical carcinoma and 31% in smears made from patients with invasive cervical carcinoma fluoresced after staining. Dyskaryotic cells taken from control subjects as well as from patients with breast cancer or cancer of the endometrium did not fluoresce after similar treatment. It seems reasonable that HSV-2 antigens or antigens that cross react with HSV-2 antigens are present in the exfoliated cells from patients with cervical carcinoma. At least part of the HSV-2 genome must be present in these epithelial cells and must be transcribed.

In another study (36) a cell line was established from biopsied cervical epithelium from a patient with cervical carcinoma. During the tenth, fifteenth and eighteenth in vitro passages, one culture out of the replicate cultures underwent spontaneous degeneration. HSV-2 was isolated from the cultures undergoing degeneration. No virus was isolated from the replicate cultures which did not undergo degeneration. The authors concluded that at least some of the cells in the original biopsy had been infected with latent HSV-2. The inability to detect virus in these cultures before they underwent degeneration argues against the idea that virus multiplication was occurring at a slow rate and indicates that virus was present in a nonreplicating form.

Attempts have been made to establish latent infections of cell cultures with HSV. Carrier state infections or persistent infections have been reported by a number of people. This type of infection is similar to the in vivo chronic infection in that virus multiplication is not completely blocked, but production of infectious virus is reduced to a low level.

Wheeler and Canby (37,38) described the establishment of a persistent infection of Hela cells with HSV. In this model system virus growth was inhibited by incorporating specific antiserum into the cell culture medium. Under these conditions the spread of the virus through the medium was inhibited and cell growth was rapid enough to keep up with cell destruction. Virus could always be recovered from the cultures and removal of the antiserum from the maintenance medium resulted in rapid virus spread and destruction of cultures.

A carrier state infection of FL cells with HSV was established by Hoggan and Roizman (39). The persistent infection in these cells was maintained by the use of antiserum and by periodically adding fresh, uninfected cells to the culture. In the course of the infection, a virus variant that produced a unique type of cytopathogenic effect was isolated. It was postulated that virus spread by 2 routes: directly from cell to cell and from cell to cell through the culture medium. Since the antiserum prevented spread of virus through the medium, the variant virus must have arisen from virus that spread directly from cell to cell.

In another study (40) Hela cells were persistently infected with HSV in the presence of antibody. Subculturing of infected cultures indicated that as few as 1 in 10^5 cells were producing virus at any one time. It was possible to isolate sublines from these cultures that were virus free. These sublines showed some increased resistance to infection although this increased resistance was not absolute.

Szanto (41) reported that a persistent infection of Hela cells could be established by incorporating 40% pooled human serum into the culture medium. Virus could be recovered throughout the experiment from the infected cultures and cytopathogenic effect could be demonstrated in localized foci. Incubation of the infected cells at 40C further reduced virus growth but did not eliminate virus from the cells.

A persistent infection of cell cultures was established by incubating the cells at a lower than normal temperature (42). Using Maben cells, a cell line derived from a lung adenocarcinoma, and a specific strain of HSV, a persistent infection was established and maintained for 9 months by incubating the cells at 31C. If the temperature of incubation was raised, the cells were readily susceptible to destruction by the virus. A number of other strains of HSV quickly destroyed the cells, even at 31C. It was postulated that a low metabolic rate in the Maben cells at 31C resulted in a slow down of virus production to a degree that was compatable with survival of the cultures.

Carrier cultures of Hela cells and KB cells were maintained over a 2 1/2 year period by the use of 30% pooled human serum (43). After 2 1/2 years two changes in the virus had occurred; it produced a modified cytopathogenic effect in cell cultures and its virulence for mice was markedly decreased.

HSV infection of a cell line derived from Chinese hamsters was studied by Hampar, Copeland and Burroughs (44, 45). During the initial passages of the newly established cell line, the cells were readily

susceptible to infection and an inoculum of any size completely destroyed the cultures. During passages 10-35, there was a successive decrease in susceptibility of the cells to the virus. The length of time it took the virus to completely destroy the cells was progressively increased. After passage 35, cell proliferation was able to keep up with cell destruction and a stable carrier culture infection was established. Cyclic changes were noted in the persistently-infected cultures. In the first part of each cycle virus titers increased and the number of viable cells decreased. In the second part of the cycle the virus titers dropped while cell proliferation resulted in growth of the cell population. This carrier culture infection differed from ones previously described in that the infection was maintained in the absence of specific environmental factors such as neutralizing antibody or deviations from optimal temperature.

Studies were carried out to determine the mechanism by which the persistent infection could be maintained in the Chinese hamster cell line. Cells from passages 20-100 were assayed for their ability to support the replication of HSV. A Hela cell-adapted strain of HSV and an early passage Chinese hamster cell-adapted strain of HSV were used. Virus was inoculated onto Chinese hamster cells of different passages and onto rabbit kidney cells. The titers of the viruses remained stable in rabbit kidney cells but dropped with each successive passage of the Chinese hamster cells. A strain of HSV isolated from late-passage, persistently-infected, Chinese hamster cells did not behave in this manner. This virus was able to replicate equally as well in Chinese hamster

cells of any passage as in rabbit kidney cells. The authors postulated that changes had occurred in both the cells and the infecting virus.

Nii (46, 47) reported the establishment of a persistent infection in Earle's L cells. This infection resembled the carrier infection in Chinese hamster cells described by Hampar, Copeland and Burroughs in that no extraneous anti-viral measures were taken in order to maintain the persistent infection. The infection was maintained over a 4 year period during which time cyclic changes in the virus titer occurred. Sublines established from cultures during periods of minimal virus growth were sometimes free of virus although virus was commonly transmitted to sublines.

Attempts were made to "cure" the persistent infection by the addition of specific antibody to the culture medium. Only a few cultures were "cured" even after continuous treatment for 1-8 months. Although a "cure" could not be affected in the majority of cultures, no evidence of virus growth was seen and no virus was recovered from supernatant fluids during treatment.

These studies in which carrier culture infections were established indicate that under well-defined conditions a chronic HSV infection at the cellular level can be maintained. It is not known, however, if this type of infection has any relation to the naturally-occurring in vivo latent infection. If the natural latent infection is really a chronic infection, it is possible that factors such as circulating antibody and local temperatures at the surface of the body limit the infection just as they do in these

carrier culture infections.

O'Neill, Goldberg and Rapp established a cell culture infection in which virus multiplication was apparently completely blocked (48). They treated human embryonic lung cells with 10-20 μg/ml of cytosine arabinoside, a potent inhibitor of DNA replication. After 4 days of treatment no infectious virus could be detected in cells that had been infected with HSV-2 at the time of treatment. Although there was no evidence of virus in the treated cultures, the virus was not eliminated from these cultures. After removal of the inhibitor from the cell culture medium, active virus growth resumed. A delay of 5-11 days between removal of the inhibitor and evidence of virus growth was observed. The authors postulated that some of the inoculum virus was able to survive during the period of treatment and resume multiplication when the concentration of the inhibitor was sufficiently reduced by periodic washing of the cultures with inhibitor-free medium. It is not possible to state what form the virus was in during this period when virus could not be detected. Presumably the block in replication occurred during the eclipse period of the replication cycle. It is unlikely that whole, infectious virus particles would be present in these cells, although virus nucleic acid must be stable.

Evidence that the virus genome is stable in cells during periods when active viral replication is not occurring comes from other studies. Duff and Rapp (33) inactivated HSV-2 by exposing extracellular virus to ultraviolet light and then infected hamster embryo fibroblasts with the

inactivated virus. Approximately 30 days after infection, foci of morphologically transformed cells were isolated and grown into cell lines. After establishment of the cell lines these cells had oncogenic potential and caused tumors when injected into baby hamsters. The transformed cells contained HSV-2 antigens as detected by immunofluorescence, but infectious HSV was not isolated from these cells. It is implied in these findings that at least part of the virus genome was stable in these cells. Failure to isolate infectious virus from these cells could indicate that the virus genome was permanently damaged, or that an exceptionally stable host-virus relationship was established.

There is evidence that at least one other HSV gene, the gene for thymidine kinase activity, can remain indefinitely in cells following insertion (49). A subline of L cells which did not contain an active gene for the production of the enzyme, thymidine kinase, was infected with ultraviolet light-inactivated HSV. After infection with the inactivated virus, colony formation occurred in medium selective for thymidine kinase positive cells. The rate of colony formation was approximately 1 out of 1000 infected cells. Assays of cell extracts for thymidine kinase activity indicated that these cells contained 7-24X as much enzyme activity as did uninfected control cells. It was proposed that infection with the ultraviolet light-inactivated virus resulted in the insertion of the virus gene for enzyme production, rather than activation of the cell gene for enzyme production. Two kinds of evidence favored this idea. The electrophoretic motility of the enzyme in the virus-infected cells

was identical to the electrophoretic pattern of the enzyme from cells lytically infected with HSV. Furthermore, a mutant of HSV which was not able to induce thymidine kinase activity during a lytic infection was not able to transform thymidine kinase negative cells into thymidine kinase positive cells following ultraviolet light inactivation.

The various clinical observations related to latent HSV infection have not fully delineated the relationship between the virus and the host at the cellular level. If anything, they have indicated that a number of cell-virus interactions are possible. Experimental studies also have not completely answered the basic questions. Clearly, further studies need to be done in order to define the nature of the host cell-virus interaction during the latent infection. Hopefully, the results presented in this study will contribute to answering some of the questions.

In an earlier study we found that when rabbit kidney cells were infected with a type 2 strain of HSV and incubated at 41C, no infectious virus was produced. When infected cultures were transferred to 37C after incubation at 41C, virus growth resumed in some of the cultures. However, a delay was observed in many cultures before virus growth occurred after transfer to 37C. During the period of time when virus growth was not detected at 37C, this infection fit the definition of a latent infection (50). We have attempted to characterize this infection, to modify the infection by treatment of the cells with various chemical agents and to adapt the infection to cell types other than rabbit kidney cells.

MATERIALS AND METHODS

Primary Rabbit Kidney Cells

Primary rabbit kidney (RK) cells were prepared according to a procedure outlined for the preparation of monkey kidney cells by Youngner (51). Kidneys were aseptically removed from freshly killed 300-600 gram New Zealand white rabbits. The fascia and capsules were stripped away from the kidneys and the kidneys were washed in Hanks' balanced salt solution (HBSS). The cortical tissue was cut away from the medullary tissue and sliced into small pieces with scissors and forceps. The tissue pieces were washed 5-6 times in cold HBSS to remove blood. The washed tissue was put into a sterile trypsinizing flask containing approximately 75 ml of 0.25% trypsin solution (Trypsin 1:250, Difco Laboratories, Detroit, Michigan) and trypsinized on a magnetic stirrer for 10-20 minutes. The trypsin was poured off and discarded. Fresh trypsin was added and the tissues trypsinized for about 30 minutes. This time the trypsin, containing the dispersed cells, was filtered through six layers of sterile gauze into sterile centrifuge tubes. Approximately 10 ml of HBSS was added to each 40 ml of cell suspension to dilute the trypsin. The cell suspension was stored at 4C. The trypsinizing procedure was repeated until essentially all of the tissue had been digested. The tubes containing the suspended cells were centrifuged at 150 xg for 15 minutes

in an International Model NH centrifuge (International Equipment Co., Boston, Mass.). The supernatant fluids were decanted and the cell pellets collected in a single tube. The cells were washed twice in HBSS and suspended in human kidney growth medium (52). Fetal calf serum (Grand Island Biological Company, Grand Island, New York) was added to the medium at a final concentration of 20%. Antibiotics (Grand Island Biological Company) were added to the medium in the following concentrations: 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone.

The cells were planted out in disposable glass cell culture tubes (Curtin Scientific Co., Minneapolis, Minn.). Each tube was inoculated with 1 ml of cell suspension containing $1\text{-}2\times10^5$ cells/ml. After 3-7 days of incubation at 37C, complete monolayers were formed. The human kidney growth medium was replaced with maintenance medium. Maintenance medium consisted of minimal essential medium (MEM) of Eagle with Earle's balanced salt solution, 2% fetal calf serum and antibiotics (53). Constituents of the medium were purchased from Grand Island Biological Company.

For certain experiments cells were planted onto glass coverslips in Leighton tubes (Curtin Scientific Co.) or into disposable tissue culture flasks (Falcon Plastics, Oxnard, California). Leighton tubes received 1.5 ml and flasks received 5 ml.

RK cells were also purchased from Grand Island Biological Company. Cells were purchased in tubes similar to the disposable tubes

used in this laboratory or in suspensions which were diluted and planted into tubes upon arrival.

RK cells prepared in this laboratory or purchased from Grand Island Biological Company were maintained in culture for up to 150 days. Cells were kept in a viable state by replacing the culture medium every 4-5 days.

Wistar-38 Cells

Wistar-38 (WI-38) cells were purchased from Grand Island Biological Company in tubes. Upon arrival at this laboratory, the cell culture medium was replaced with our maintenance medium (MEM) and the cells were ready to use. These cells were kept viable by changing the culture medium every 4-5 days.

Human Kidney Cells

Human kidney (HK) cells were purchased in tubes from Microbiological Associates (Bethesda, Maryland). After arrival in the laboratory, these cells were treated in the same manner as were the RK cells and the WI-38 cells.

Virus

Herpes simplex virus type 2 (strain MS) ATCC VR-540 was purchased from the American Type Culture Collection and used throughout these studies. Stock cultures of virus were prepared by growing the virus in 60 cm^2 bottles of RK cells. A large inoculum of virus

(approximately $1x10^5$ infectious virus particles) was added to fresh cultures of cells. When the monolayers were approximately 3/4 destroyed, the cells were frozen and thawed 3 times to release intracellular virus. After clarification by centrifugation at 150 xg for 5 minutes, the supernatant fluid containing the virus was stored in 1 ml quantities at -90C in a Revco ultra-low temperature freezer (Revco, Inc., Deerfield, Michigan). Stock cultures of virus containing 10^6-10^7 infectious virus particles/ml were obtained in this way.

Detection of Virus Growth

Detection of virus growth was accomplished by observing infected cultures for the characteristic pattern of cell destruction that HSV is known to induce. HSV tends to spread directly from cell to cell and focal areas of cell destruction are seen early in the infection. These focal areas of infection are characterized by the presence of swollen, rounded, refractile cells and occasional multinucleated giant cells. These focal areas expand in size as the infection progresses. At the same time some infected cells detach from the monolayer and spread virus through the supernatant fluid so that new focal areas arise. Once evidence of virus growth is observed, the destruction of the entire culture is completed within a few days. Virus-induced cell destruction is termed cytopathogenic effect (CPE) and will be referred to as such.

Quantitation of Virus Growth

Two standard assay procedures were used for the quantitation of infectious virus in these studies. The plaque assay method was used for precise quantitation of virus. The tissue culture infectious dose-50% (TCD_{50}) was used to determine the inoculum size in most experiments. In addition, a modification of this assay was used to determine virus survival in viable cells. In the routine assays, cultures to be assayed for virus were treated by freeze-thawing 3 times in order to release intracellular virus. Certain cultures were treated by sonication at 10,000 cycles/second prior to assay.

Plaque Assay--The plaque assay method is a quantitative assay procedure in which the actual number of virus particles that are capable of initiating infection are counted. Infectivity is expressed as plaque forming units (PFU) of virus. In this procedure 10-fold dilutions of virus were made in HBSS. Duplicate cultures of RK cells containing 2 ml of HBSS were inoculated with 0.2 ml of each dilution of virus. After a 4 hour adsorption period at 37C, the supernatant fluids were decanted and the monolayers overlayed with a solution of maintenance medium (MEM) containing 1.5% methylcellulose and incubated at 37C. The MEM + methylcellulose solution was prepared according to a method described by Dolan et al. (53). Methylcellulose (400 centipoise) was purchased from Fisher Scientific Company (Fairlawn, New Jersey). After 5 days of incubation at 37C, the semi-solid medium was decanted. Absolute

methanol (Mallinckrodt Chemical Works, St. Louis, Missouri) was added to cover the monolayers and allowed to remain in contact with the cells for 10 minutes. After 10 minutes, the alcohol was removed and the monolayers were stained with a 1:10 dilution of Giemsa blood stain (Hartman-Leddon Company, Philadelphia, Pennsylvania). After 20 minutes, the staining solution was removed. In this procedure the cell sheet stained dark purple and plaques were seen as colorless holes in the monolayers. Both flasks and tubes of cells were used in this assay method.

TCD_{50} Assay--The TCD_{50} assay is a quantal assay and the results are determined on the basis of the infectivity endpoint following serial dilution. Ten-fold dilutions of virus were made in HBSS. Duplicate tube cultures of RK cells were inoculated with 0.1 ml of each dilution. Cultures were incubated at 37C for up to 1 week with daily microscopic observation for CPE, although in all cases, CPE was seen within 3 days when the sample did contain virus. The TCD_{50} value was calculated from the infectivity endpoint by the Reed-Muench formula (54).

The plaque assay method is definitely more precise than the TCD_{50} assay because the actual number of virus particles that are able to initiate infection are counted rather than estimated. Furthermore, the plaque assay is only slightly more involved than the TCD_{50} assay and can be run with fewer cultures. The TCD_{50} assay is useful for determining if any virus is present at all in a sample. However, in a matched experiment, it proved to be only slightly superior to the plaque assay

method in detecting endpoint virus.

A modification of the TCD_{50} assay proved to be extremely useful as an assay for virus in viable cells. In this viable cell assay, the cells to be assayed for virus were removed from the monolayers by treatment with 0.25% trypsin. The dispersed cells were centrifuged at 150 xg for 10 minutes to separate them from the trypsin. The supernatant fluids were decanted and the cells were resuspended in human kidney growth medium. Ten-fold dilutions of the cells were made in growth medium. Cells from each dilution were added to full monolayers of RK cells which served as a substrate upon which the added cells could attach. These carrier monolayers of cells had to be used because the suspended RK cells did not form colonies when incubated in tubes at low concentrations. Furthermore, the carrier monolayers served as efficient indicator cells which were readily infected once active virus growth occurred. Since virus growth often did not occur for some time after transfer of the viable cells to the carrier cultures, it was not possible to use a plaque assay procedure in this case.

Neutralization of Virus

Virus isolates were identified as HSV on the basis of a neutralization test. Stock cultures of the virus isolate were diluted in HBSS to contain a final concentration of approximately $1x10^3$ PFU/ml. Anti-HSV serum prepared in guinea pigs (Microbiological Associates) was diluted 1:64, and 0.5 ml of this dilution of serum was mixed with 0.5 ml of the

diluted virus. After standing at room temperature for 1 hour, 0.2 ml of the virus-serum mixture was inoculated into duplicate cultures of RK cells. Cultures were then incubated at 37C along with cultures that had been infected with virus that had been incubated with HBSS rather than serum. Cultures were observed daily for CPE. The test was terminated when cultures that had been infected with control virus showed CPE. Inhibition of virus by the anti-HSV serum was accepted as confirming the isolate to be HSV.

High-Temperature Incubation

Cells were incubated at 41C in a water-jacket-regulated incubator (National Appliance Company, Portland, Oregon). The temperature was found to vary not more than $\pm$0.5 degrees. The temperature of the incubator was set at 41.5C so that the minimum temperature would fall to no lower than 41.0C.

Fluorescence Microscopy

Acridine orange (AO) staining and immunofluorescence staining were used to visualize the virus-infected cells. For these studies cells were grown on glass coverslips in Leighton tubes.

Acridine orange--A procedure described by Gluck and Kulovich was followed for staining monolayers with acridine orange (AO) (55). The preparation of reagents has been described by these authors. Stock solutions of reagents were prepared in advance and stored at room

temperature or at 4C. AO was purchased from Matheson, Coleman and Bell (Norwood, Ohio). A 0.1% solution was prepared in glass-distilled water and stored at 4C. Carnoy's solution was prepared fresh each time it was used. The actual staining procedure that was followed is shown in Table 1.

It was not necessary to complete the staining procedure immediately once it was begun. Coverslips were often kept for 1-5 days in Carnoy's solution. All biological activity within the cells was halted in this solution. When the staining procedure was completed, the coverslips were mounted face down on clean glass slides using a few drops of phosphate buffer as the mounting fluid. Slides were ready for viewing at this time but could be kept for up to 2 weeks in a dark, humidified container at 4C, without fading.

Microscopy was carried out using an Ortholux microscope (Ernst Leitz, Inc., Rockleigh, New Jersey). The microscope was equipped with a 200W high-pressure mercury vapor lamp to provide an untraviolet light spectrum. A dark-field condenser was used at all times. The following filter combination was used for viewing AO-stained slides: KG1, BG12, BG38, and K530 filters. The KG1, BG12 and BG38 filters were situated between the ultraviolet light source and the specimen; the K530 filter was situated between the specimen and the eye piece or photographic plate. The KG1 filter was used to adsorb out most of the radiation of the infrared spectrum and protect the other filters from excess heat. The BG12 excitation filter adsorbed out most visible light while allowing

TABLE 1

ACRIDINE ORANGE STAINING PROCEDURE

Reagent	Time	Comments
PBS	30 seconds	with rinsing
PBS	30 seconds	with rinsing
PBS	30 seconds	with rinsing
Carnoy's solution	5 minutes	
95% ethyl alcohol	30 seconds	
80% ethyl alcohol	30 seconds	
70% ethyl alcohol	30 seconds	
50% ethyl alcohol	30 seconds	
0.002M $MgSO_4$	30 seconds	with rinsing
1% acetic acid	1 minute	
0.002M $MgSO_4$	30 seconds	with rinsing
0.006M Phosphate buffer	30 seconds	with rinsing
0.01% acridine orange	5 minutes	
0.067M Phosphate buffer	30 seconds	with rinsing
0.067M Phosphate buffer	5 minutes	
0.01M $CaCl_2$	2 minutes	
0.067M Phosphate buffer	30 seconds	with rinsing

transmission of light in the ultraviolet region of the spectrum. The BG38 red-suppression filter was used to adsorb the small amount of visible light in the red region of the spectrum which was not adsorbed by the BG12 filter. Ultraviolet light which was not adsorbed by the glass slide and specimen was adsorbed by the K530 filter. This filter allowed only visible light with a wave length of longer than 530 nm to be transmitted. Slides were viewed under oil immersion at a magnification of 540X.

Photomicrographs were made using the Orthomat microscope camera (Ernst Leitz, Inc.). Exposure times varied from approximately 20 seconds to 2-3 minutes.

Indirect Immunofluorescence--Indirect fluorescent antibody staining was done according to a method outlined by Ross, Watson and Wildy (56). Specific antiserum to HSV was prepared in a 2000 gram, male, New Zealand, white rabbit. A 20 in^2 area of the animal's back was shaved and scarified. A stock culture of infectious virus containing approximately 10^5 PFU of virus in 1 ml of maintenance medium was applied to the scarified skin with a cotton swab. Herpetic lesions appeared at the inoculation site after 7-10 days. The lesions healed in about 10 days and the animal suffered no other obvious sequelae, although other animals treated in the same manner died following the initial infection. Three subcutaneous injections consisting of 1 ml of stock virus mixed with 1 ml of Freunds complete adjuvant (Difco Laboratories) per injection were given to the rabbit on three consecutive weeks. Fifty ml of blood

were removed aseptically by cardiac puncture 10 days after the final injection. The serum was separated from the blood cells by centrifugation and stored at -20C in 1 ml quantities. A 1:40 dilution of serum was able to neutralize 100 PFU of virus in a standard neutralization test.

Serum prepared in sheep against rabbit serum and conjugated to the fluorescent dye, fluoresceinisothiocyanate (FITC) was purchased from Grand Island Biological Company in lyophilized form. The serum was reconstituted with distilled water and stored at -20C in 0.5 ml quantities. Once thawed, the serum that was not used was discarded.

Cells grown on glass coverslips in Leighton tubes were washed 3 times with phosphate-buffered saline (PBS) and fixed in acetone for 30 minutes at -20C. After fixation in acetone, the cells were again washed 3 times in PBS. Cells were ready for staining at this point. They were stained immediately or left in the third PBS wash until they were stained.

Coverslips to be stained were placed cell-side-up on a moist paper towel. Rabbit anti-HSV serum was diluted 1:2 with PBS. Enough serum was added to the coverslips to cover the surfaces. Surface tension kept the serum from running off. The coverslips were then placed at 37C for 30 minutes. If the serum began to dry, more serum was added. After staining for 30 minutes, they were washed 3 times in PBS. Following the third wash, the coverslips were stained with sheep antirabbit serum that was diluted 1:2. Staining with the sheep serum was done in exactly the same manner as the staining with the rabbit serum. After

being stained with the FITC-conjugated anti-rabbit serum, the coverslips were again washed 3 times in PBS. After the third wash, they were mounted cell-side-down on clean glass slides using a 10% PBS-90% glycerol solution for mounting.

Preparations were viewed by dark-field microscopy with the Ortholux microscope. A filter combination consisting of a KG1 heat barrier filter, UG1 excitation filter, BG38 red suppression filter and K430 barrier filter was used. Stained preparations were viewed under visible light and under ultraviolet light. Fluorescence was obtained with either light source. Under visible light the staining was much brighter. However, there was a certain amount of background fluorescence. Under ultraviolet light there was very little background fluorescence and the differential staining was much more obvious. Photomicrographs were taken with the Orthomat camera.

Preparation of Chemical Reagents

Various chemical reagents were used in certain studies. Chemicals were purchased from Sigma Chemical Company (St. Louis, Missouri) in dried form. Stock solutions were prepared either in HBSS or in 95% ethanol and sterilized by filtration through swinnex filters with 300 nm pore size. Filter membranes were obtained from the Millipore Corporation (Bedford, Mass.). The various chemical reagents used along with the solvents in which each was dissolved and the concentrations of stock and working solutions are listed in Table 2.

TABLE 2

PREPARATION OF CHEMICAL REAGENTS

Chemical	Solvent	Concentration of stock solution (µg/ml)	Concentration of working solution (µg/ml)
Hydrocortisone	95% ethyl alcohol	1000	2.0- 25
Progesterone	95% ethyl alcohol	1000	0.5- 5.0
17B estradiol	95% ethyl alcohol	1000	0.5- 5.0
L-thyroxin	95% ethyl alcohol	400	0.2- 2.0
L-epinephrine	HBSS	1000	0.1- 1.0
BUDR	HBSS	1000	25 -100
IUDR	HBSS	1000	25 -100
Puromycin	HBSS	500	5 - 20

Information regarding solubility and other chemical properties of these chemicals was obtained from the book, Data for Biochemical Research (57). L-epinephrine was soluble in HBSS at pH 5. The pH was adjusted with 0.1 N HCl. No precipitation was observed when the pH of the working solution was readjusted to pH 7.2. L-thyroxin (thyroid powder) and L-epinephrine were prepared fresh for each experiment. Stock cultures of the steroid hormones were stored at 4C. Fresh solutions were prepared for each experiment. The Pyrimidine, 5-iodo-2-deoxyuridine (IUDR) dissolved very slowly in HBSS. Solubilization was obtained by reducing the pH of the solution to pH 5 with 0.1 N HCl and warming the solution in a water bath. After 6-8 hours no undissolved IUDR remained. Stock solutions of IUDR, 5-bromo-2-deoxyuridine (BUDR) and puromycin were kept at -20C for 2-3 months.

Photosensitization Studies

Neutral red dye was purchased from Grand Island Biological Company as a 1:300 dilution of filter-sterilized dye. Dye was added to the cell culture medium at concentrations of 1:240,000-1:480,000. After exposure to the neutral red, cells were exposed to visible light from a 30W fluorescent light bulb at a distance of approximately 20 cm.

RESULTS

Influence of Incubation Temperature on the Growth of HSV-2

Previously it had been shown that the MS strain of HSV-2 did not grow in RK cells at 41C (50). Cultures had been infected with 500 TCD_{50} of virus. Since tube cultures contained approximately $1x10^6$ cells, this inoculum amounted to a multiplicity of infection of about 1 infectious virus particle per 2000 cells. No CPE was observed in these cultures after incubation at 41C for 1-5 days. On each day duplicate cultures were freeze-thawed 3X and assayed for virus. No infectious virus was detected in any of the cultures treated in this way. In subsequent work, 40 cultures were treated by sonication at 10,000 cycles/second for 30 seconds at the time of assay for virus. No virus was detected in these cultures. Control cultures were infected with the same amount of virus and incubated at 37C. CPE was seen in these cultures as early as 24 hours after infection, and infected cultures were completely destroyed by the third day. High titers of infectious virus were recovered from these cultures. As much as $1x10^7$ PFU/ml were recovered on days 2 and 3.

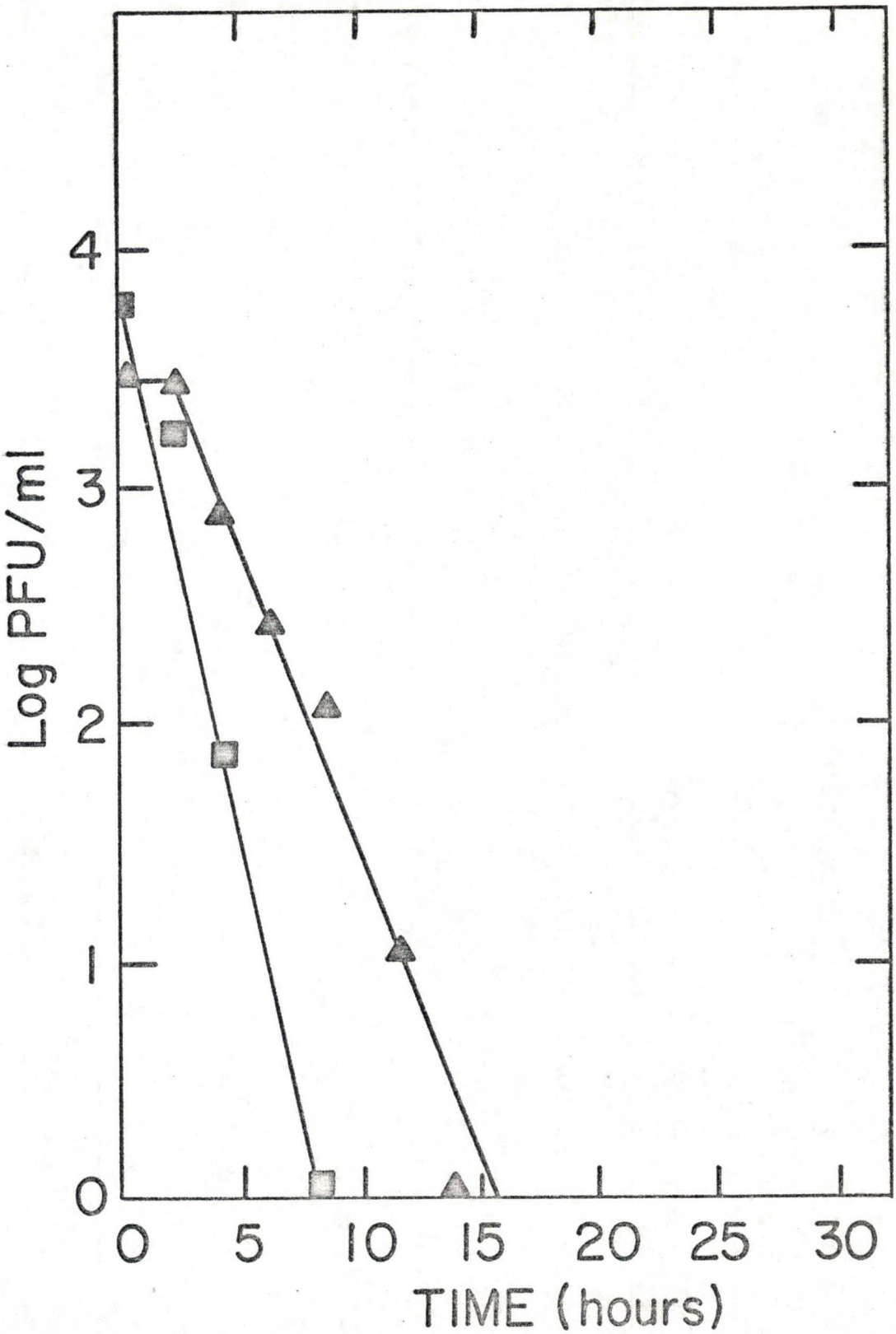
A study was devised to determine the rate of virus inactivation at 41C. Cultures of RK cells were infected with virus and incubated at 41C.

At 2-hour intervals duplicate cultures were freeze-thawed 3X and assayed for virus in RK cells. The rate of inactivation is shown in Figure 1. Virus disappeared at a logarithmic rate. The half-life of the virus was approximately 100 minutes and 99% of the virus was inactivated in about 12 hours. No virus was detected in any culture that had been incubated at 41C for 15 hours or longer when the cultures were disrupted by freeze-thawing at the time of assay.

The rate of inactivation of virus at 41C in an environment devoid of viable cells is also shown in this Figure. In this experiment virus was added to cultures of RK cells that had been previously disrupted by freeze-thawing. These cultures were then treated in exactly the same manner as were the viable cells referred to previously. It can be seen that virus inactivation was even more rapid in a cell-free environment than in viable RK cells. The half-life of the virus in the viable cell-free environment was approximately 36 minutes and 99% of the virus was inactivated in 4 hours.

Effect of 41C Preincubation on the Ability of RK Cells to Support Exogenous Virus Replication After Transfer to 37C

Since virus growth was markedly inhibited in cells incubated at 41C, it was necessary to determine if incubation at 41C adversely affected the cells and prevented them from supporting virus replication. If the cells were adversely affected by incubation at 41C, it would be expected that replication of exogenously added virus would be inhibited at 37C, at


Log PFU/ml
0
1
2
3
4
0
5
10
15
20
25
30
TIME (hours)

least temporarily, in cells preincubated at 41C. The ability of cells that had been preincubated at 41C to support the replication of exogenous virus after transfer to 37C was therefore measured. Two parameters of virus growth: 1) the ability of virus to adsorb to cells and 2) the ability of cells to produce infectious virus, were measured. The amount of virus able to adsorb to cells was determined by adding a stock culture of HSV, diluted to contain approximately 500 PFU of virus, to cells that had been preincubated at either 37C or at 41C for 2 days. After an adsorption period of 2 or 4 hours, the supernatant fluids were decanted and the cells were washed and overlayed with the methylcellulose-containing maintenance medium. These cultures were then incubated at 37C for 4 days and plaques were counted at this time. The amount of virus that adsorbed to cells preincubated at 41C was comparable to the amount that adsorbed to cells preincubated at 37C (Table 3).

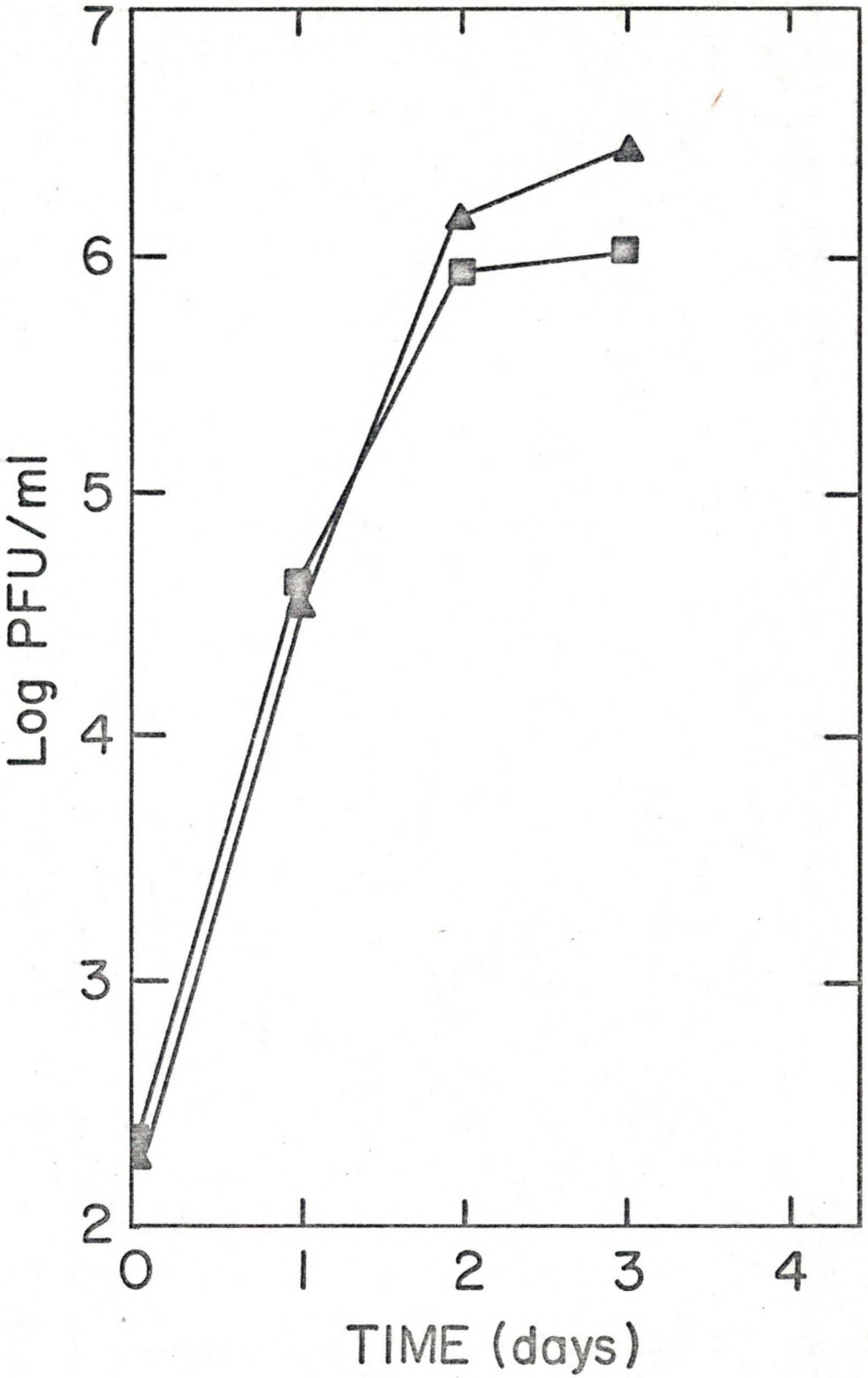
To test the ability of cells to produce infectious virus after preincubation at 41C, tube cultures containing approximately $1x10^6$ RK cells were infected with 500 PFU of virus and incubated at 37C. Cultures preincubated at 37C served as controls. At 24, 48 and 72 hours after infection cultures were freeze-thawed 3X and assayed for infectious virus. There was no significant difference in the amount of virus produced in cultures preincubated at 41C as compared to cultures preincubated at 37C (Figure 2).

TABLE 3

ADSORPTION OF HSV TO RK CELLS PREINCUBATED AT 37C OR AT 41C FOR 2 DAYS

Preincubation temperature	Adsorption period (hours)	Amount of virus adsorbed to cells (PFU/ml)
37C	2	330
41C	2	300
37C	4	628
41C	4	760

NOTE: RK cells were preincubated at either 37C or 41C for 2 days. They were then infected with a stock of HSV. Virus was allowed to adsorb to cells for 2 or 4 hours at 37C. Following the adsorption period, unadsorbed virus was decanted and the cells were washed in HBSS. After washing, the cells were overlayed with methylcellulose-containing medium and incubated at 37C for 4 days. At the end of 4 days, the cultures were stained with Giemsa stain and plaques were counted.


7
6
5
4
3
2
Log PFU/ml
0
1
2
3
4
TIME (days)

Survival of HSV-2 at 41C; Replication after Transfer to 37C

Although virus could not be recovered from freeze-thawed or sonicated cultures during incubation at 41C, the virus was not irreversibly inactivated. Virus was recovered from cultures incubated at 41C after they were transferred to 37C in a viable condition and allowed to continue incubating at 37C. In some cultures evidence of virus growth was seen very quickly after transfer to 37C. In other cultures there was a delay before evidence of virus growth was observed. The first sign of active virus growth was the formation of the typical foci of rounded cells that is characteristic of HSV-induced CPE. Once CPE appeared it always progressed with the whole culture being destroyed within a few days. At the earliest evidence of CPE assay of the supernatant fluid for infectious virus showed no virus or only a very small amount. However, virus could always be recovered from freeze-thawed cells at this time.

The period of time between transfer of cultures from 41C to 37C and the appearance of CPE was defined as the lag period. When exogenous virus was inoculated directly onto cells at 37C, CPE was always seen within 3 days. Therefore, lag periods of 4 days or longer indicated that there was a delay before virus replication began at 37C. Table 4 shows the data obtained from experiments in which 200 cultures were incubated at 41C for varying lengths of time and then transferred to 37C. These cultures were infected with between $1x10^3$-$1x10^4$ TCD_{50} of virus at the start of incubation at 41C.

TABLE 4

RECOVERY OF HSV AT 37C AFTER INCUBATION AT 41C IN RK CELLS

Number of cultures incubated at 41C	Incubation period at 41C (days)	Number of cultures in which virus was recovered after incubation at 37C		
		for 1-3 days	for 4-6 days	for more than 6 days
34	1-2	32	0	0
166	3-7	54	34	48

NOTE: Cultures were infected with $1x10^3$-$1x10^4$ TCD_{50} of virus and incubated at 41C. Cultures were transferred from 41C to 37C on each of the next 7 days. Cultures were allowed to continue incubating at 37C and were monitored daily for CPE.

Thirty-four cultures were incubated at 41C for 1-2 days. CPE was observed in 32 of these cultures within 3 days after transfer to 37C. Of the 166 cultures incubated at 41C for 3-7 days, virus was recovered from 54 of these cultures within 3 days. In 82 cultures from this group virus was recovered after lag periods longer than 3 days. Thirty-four cultures had lag periods of 4-6 days and 48 cultures had lag periods in excess of 6 days. The average lag period of cultures in this last group was 15.3 days and the longest lag period observed was 45 days. Overall, virus was eventually recovered from 168 of the 200 cultures initially infected for a recovery rate of 84%. Eighty-two of these 200 cultures (41%) had lag periods in excess of 3 days and 48 cultures (24%) had lag periods of longer than 6 days. In these studies the cultures in which virus did not grow were routinely incubated for 45-60 days at 37C before being discarded. Some cultures were incubated as long as 150 days.

Determination of the Amount of Virus Surviving Incubation at 41C

Once it became apparent that some of the inoculum virus was able to survive incubation at 41C for an extended period of time in viable cells, attempts were made to quantitate this virus. One method for quantitating virus survival involved using a viable cell assay as described in the Materials and Methods section of this paper. This method involved removing monolayers of infected cells from 41C incubation, dispersing the monolayers into individual cells and adding specified numbers of these cells to carrier cultures of RK cells. The carrier cultures

of RK cells were then incubated at 37C and monitored for CPE. Infected cells incubated at 37C for 6 hours were treated in exactly the same manner and served as controls. The results of this experiment are shown in Table 5. Virus was transferred to carrier monolayers with as few as 9 cells from the control cultures incubated at 37C for 6 hours. Virus was transferred with $9x10^2$ cells from 7 of 12 cultures that had been incubated at 41C for 2, 4 or 6 days. No virus was transferred with $9x10^1$ or 9 cells from these cultures. This indicated that as much as 1% of the inoculum virus remained viable during incubation at 41C.

Carrier cultures which received cells that had been incubated at 37C showed CPE within 3 days. In carrier cultures that received cells from 41C incubation, extended lag periods occurred in many cultures before CPE was seen. Since virus growth occurred after widely-varying lengths of time in these cultures, it was not practical to quantitate virus by plaquing in this situation.

In another experiment tube cultures were infected with concentrations of virus ranging from 4000 PFU/culture to 4 PFU/culture and incubated at 41C for 2, 4 or 6 days. After incubation at 41C, cultures were transferred to 37C and continued incubating at this temperature. Cultures at 37C were monitored daily for CPE (Table 6). Virus was eventually recovered from 19 of 24 cultures that had received 4000 PFU of virus and from 15 of 24 cultures that had received 400 PFU. Virus was only recovered from 3 cultures that had received 40 PFU and from no cultures that had received 4 PFU of virus. This indicated that a minimum of

TABLE 5

AMOUNT OF VIRUS SURVIVING INCUBATION AT 41C

Incubation conditions of infected cells	Number of carrier cultures in which virus was recovered after the addition of the following number of cells from infected cultures				
	$9x10^4$	$9x10^3$	$9x10^2$	$9x10^1$	$9x10^0$
6 hours at 37C	4/4	4/4	4/4	4/4	2/3
2 days at 41C	4/4	3/4	3/4	0/4	0/4
4 days at 41C	4/4	2/4	3/4	0/4	0/4
6 days at 41C	4/4	3/4	1/4	0/4	0/4

NOTE: Cultures were infected with HSV and incubated at 37C for 6 hours or at 41C for 2, 4 or 6 days. After incubation for the appropriate length of time, the cells were dispersed with trypsin, centrifuged and resuspended in growth medium. Serial dilutions of the cells were made and carrier cultures at RK cells were inoculated with each dilution. Ten-fold dilutions of cultures originally containing $1x10^6$ cells gave the final concentrations of cells shown here. Carrier cultures were incubated at 37C and monitored for CPE.

TABLE 6

AMOUNT OF VIRUS NEEDED TO ESTABLISH A LATENT INFECTION

Length of the incubation period at 41C (days)	Number of cultures (out of 8) in which virus was recovered at 37C after incubation at 41C with the following amount of virus (PFU/culture)			
	4000	400	40	4
2	7/8	7/8	2/8	0/8
4	6/8	5/8	0/8	0/8
6	6/8	3/8	1/8	0/8

NOTE: Twenty-four cultures of RK cells were infected with each dilution of virus and incubated at 41C. After 2, 4 and 6 days cultures were transferred to 37C and incubated at this temperature. Cultures were monitored daily for CPE. Values for the inocula were determined by simultaneously assaying the virus in RK cells.

0.25% of the inoculum virus survived incubation at 41C.

Investigations into the Stage at which Virus Replication was Blocked

Attempts were made to determine the stage at which virus replication was blocked. Previously it was shown that virus was able to adsorb to cells as efficiently at 41C as at 37C (50). This was expected because if replication were blocked previous to this step, whole infectious virus would have had to be present and none was found.

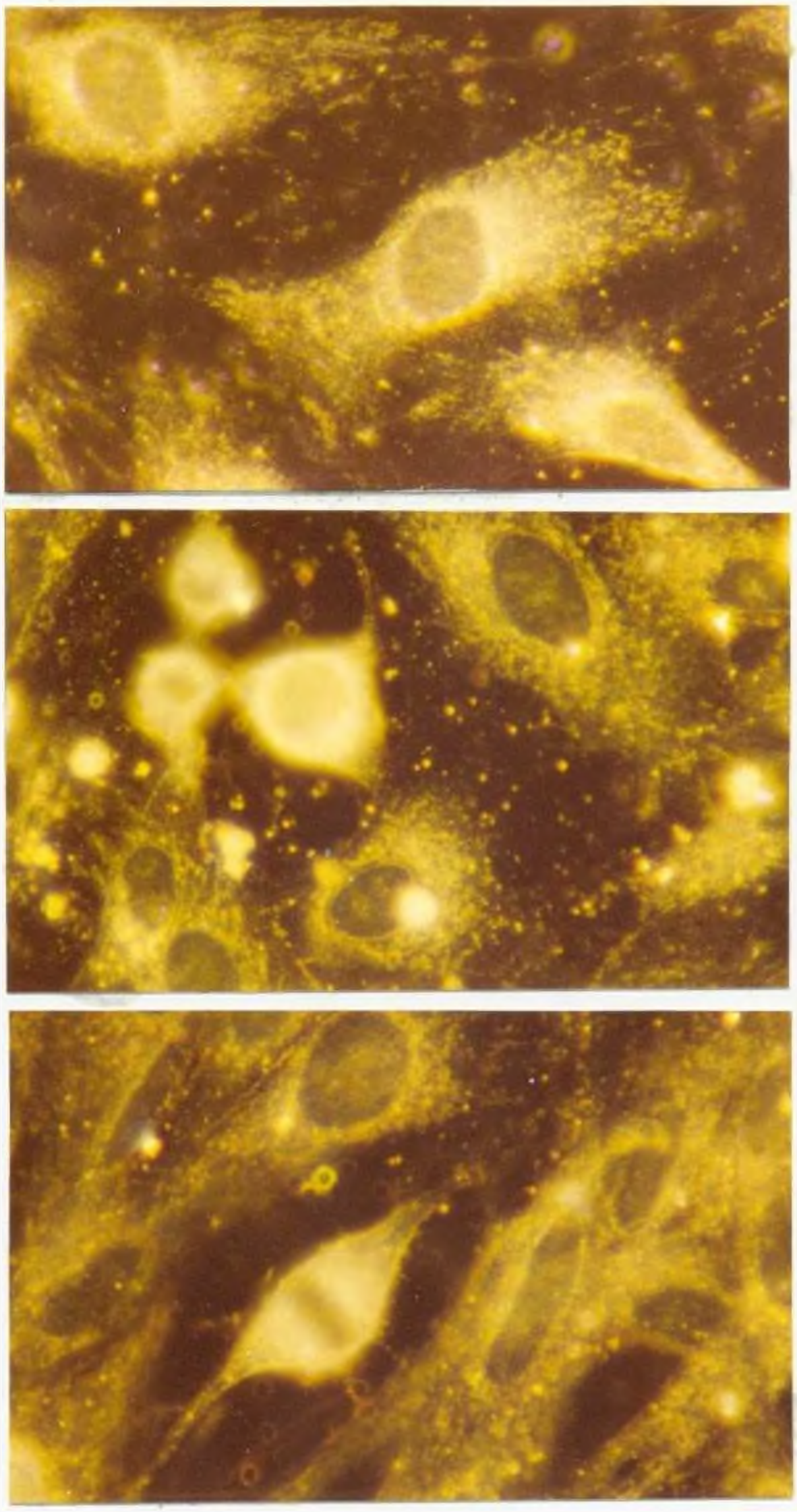
Immunofluorescence studies were initiated to determine if virus-induced antigens were produced in cells infected at 41C. It was necessary to first determine at what stage in the normal viral replication cycle at 37C that virus antigens appeared. Cultures of RK cells were grown on glass coverslips in Leighton tubes and infected with approximately $1x10^5$ PFU of virus (multiplicity of infection of 1 infectious virus particle per 10 cells). After incubation at 37C for 2, 4, 6, 8 and 24 hours cultures were fixed in acetone, stained by the indirect immunofluorescence technique as described in the Materials and Methods section of this paper and examined for the presence of virus-specific antigens. Uninfected control cultures were also treated the same way and examined.

A certain amount of background fluorescence was present in control cultures. This fluorescence was localized in the cytoplasm of the cells and was very diffuse. The nuclei of uninfected cells were conspicuous by their lack of fluorescence.

Cultures that were incubated for 2 hours at 37C appeared indistinguishable from control cultures. By 4 hours after infection it was possible to detect fluorescence in the nuclei of a few cells. Perinuclear cytoplasmic fluorescence could also be detected in some cells by this time. In the 4-hour cultures the nuclei were still distinct from the cytoplasm. In cultures incubated for 6 hours and 8 hours after infection many cells showed definite fluorescence. In these cells whole nuclei often fluoresced. Some of the cells were rounded up, a characteristic feature of end-stage HSV infection. The nuclei of these cells were not distinct and whole cells often fluoresced brightly. Cells from cultures that had been incubated for 24 hours were identical to cells from the 8-hour cultures. The infection process, however, involved nearly all of the cells in the 24-hour cultures. Control cells and cells infected at 37C are shown in Figure 3.

Tube cultures were infected with the same amount of virus ($1x10^5$ PFU/culture) and incubated at 41C for 1, 2, 3 and 4 days after which they were stained for virus-specific antigens. Most of the cells in these cultures looked identical to uninfected control cells after being stained for virus antigens. There were, however, isolated cells which showed intranuclear or perinuclear fluorescence. In some cells the entire nuclei fluoresced. Isolated fluorescing cells could be seen in cultures incubated for 1, 2, 3 or 4 days at 41C. Cells from a culture incubated for 3 days are shown in Figure 3.

Fig. 3. Monolayers of RK cells stained by the indirect immunoflourescence method. The cells in the top photograph were not infected with virus and only background fluorescence can be seen. The cells in the middle photograph were incubated at 37C for 8 hours after infection with HSV. Fluorescing and nonfluorescing cells can be seen. The cells in the bottom photograph were incubated at 41C for 3 days following infection. One fluorescing cell can be seen. Magnification is 540X.



A study was done to determine the number of infected cells that had to be present before fluorescing cells could be readily detected. A stock culture of virus was diluted in HBSS to contain between $1x10^5$-$1x10^1$ PFU per 0.2 ml. Coverglass cultures were infected with 0.2 ml of each dilution. The virus was assayed simultaneously in RK cells. Cultures were stained for virus-specific antigens after 1 day of incubation at 41C. Fluorescing cells were readily seen in cultures that received $1x10^3$ or more PFU of virus. Fluorescence could be seen in cultures that received $1x10^2$ PFU but since there always was a certain amount of background fluorescence it was not easy to differentiate quantitatively between these cultures and uninoculated control cultures.

When very few cells in a culture were infected, it was not possible to detect morphologic alterations by bright field microscopy. CPE was noted only after small foci of infected cells developed. Staining with acridine orange (AO) proved to be useful in identifying morphologic alterations in infected cells even when only isolated infected cells were present. This technique was used to monitor cells productively infected with HSV at 37C and cells infected with virus at 41C. Control cultures and cultures infected with $1x10^5$ PFU of virus were incubated at 37C for 1, 2, 4, 6, 8 and 24 hours. At each time period cultures were removed from incubation, fixed, and stained with AO as described in Materials and Methods.

The nuclei of control cells stained brightly green while the cytoplasm fluoresced with an orange color. The nuclei of these cells did not

stain uniformly. Patches of very intense-staining material were seen against a background of moderately-staining material. The green fluorescence of the nuclei was due to the binding of dye to DNA. It is probable that the intense-staining material were deposits of heterochromatin. The areas of intense-staining material were scattered randomly throughout the nuclei. In addition, most of the nuclei contained 1 or more small, round areas of orange-fluorescing material. These were presumed to be nucleoli.

Cultures incubated at 37C for 1, 2 or 4 hours after infection were identical to control cultures. The first morphologic alterations in the nuclei of these cells were visible after 6-8 hours of incubation. The earliest observable change in the nuclei was the formation of a bright ring of fluorescence around the periphery of the nucleus accompanied by a decrease in fluorescence in the center of the nucleus. Possibly the formation of the ring at the periphery was due to the DNA in the cell being pushed to the periphery in the course of the infection. This characteristic feature was referred to as "margination of the chromatin." Two additional alterations were noted in cultures incubated for 8 hours or longer. The overall intensity of the nuclear staining increased. The nuclei of infected cells were almost yellow in contrast to the green color of uninfected nuclei. In addition to these changes in the nuclei, cells incubated for 8 hours or longer began to lose their normal spindle shape and started to round up. In cultures incubated for 24 hours, the infection had spread to most of the cells in the cultures. Individual

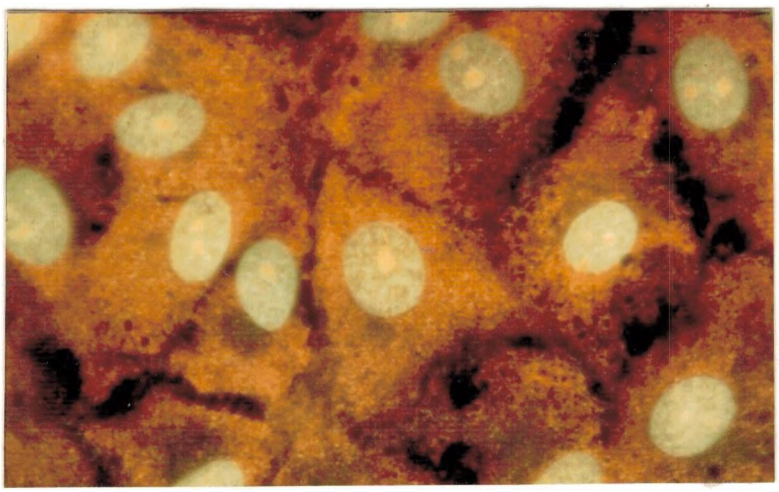
cells were similar to cells from 8-hour cultures although some multinucleated giant cells could be seen. Cells from a control culture and a 24-hour culture are shown in Figure 4.

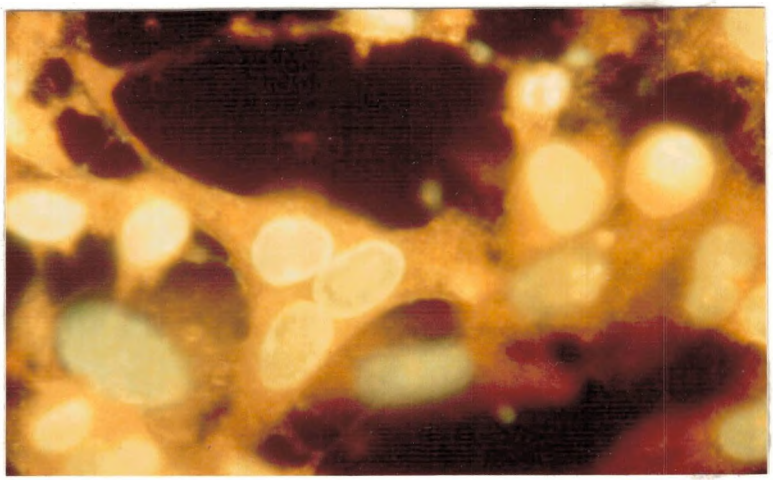
The sensitivity of the AO staining technique in identifying infected cells was determined. Cultures of RK cells were infected with dilutions of virus containing from $1x10^5$-$1x10^1$ PFU and incubated for 10 hours at 37C. After 10 hours of incubation, the cultures were stained with AO and observed. Infected cells were readily identified in cultures that had received $1x10^2$ or more PFU of virus. Infected cells could be seen in cultures infected with less than $1x10^2$ PFU, but unless the slides were closely scrutinized, the few infected cells were often missed.

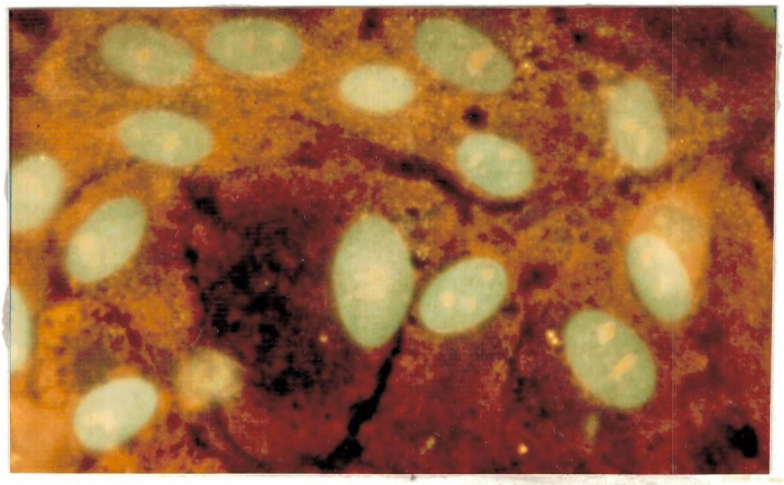
Cells incubated at 41C were also followed by AO staining. Cultures were infected with $1x10^5$ PFU of virus and incubated at 41C for 1, 2, 3 or 4 days. Cultures from 41C incubation appeared identical to uninfected control cultures. There was no evidence of "margination of the chromatin" and the cells retained their spindle-shaped morphology. Infected cells from a culture incubated at 41C for 4 days are shown in Figure 4.

Attempt at Establishing a Latent Infection By Infecting Cells at 37C with a Low Concentration of Virus

With certain assay procedures, when a low concentration of virus is used as the inoculum, there is a very long lag period before evidence of virus growth (58). It was possible that in our experiments







nearly all of the inoculum virus was inactivated at 41C and that the extended lag periods were the result of a normal delay that accompanies infection with a very small amount of virus. If this were true, however, the same phenomenon should occur at 37C when a very small concentration of virus was used as the inoculum. To test this, a stock culture of virus was serially diluted and inoculated onto RK cells at 37C (Table 7). Eighteen cultures were infected with each dilution of virus. Cultures were monitored daily for CPE. In all cultures which developed CPE, evidence of virus growth was visible within 3 days. If CPE were not seen within 3 days, further incubation for up to 30 days resulted in no new development of CPE. It was not possible to establish a latent infection in this manner.

Attempts at Modifying the Latent Infection by Treatment of Cell Cultures with Various Hormones

Attempts were made to modify the latent infection by treatment of cell cultures with various hormones and certain other chemical agents. In some studies the chemical compounds were incorporated into the cell culture medium either prior to infection with virus or at the time of infection with virus and incubation at 41C. In later studies cultures were also treated with chemical agents at the time of transfer from 41C to 37C. Treatment of cultures prior to infection or at the time of infection with virus was done to determine if it were possible to either prevent the formation of the latent infection or to enhance the establishment of the latent infection. Treatment of cultures after infection with virus

TABLE 7

ATTEMPT AT ESTABLISHING A LATENT INFECTION IN RK CELLS AT 37C BY INOCULATING CULTURES WITH VERY LOW CONCENTRATIONS OF VIRUS

Amount of virus inoculated (TCD_{50})	Number of cultures inoculated	Number of Cultures Showing CPE within 3 days	Number of Cultures Not Showing CPE within 3 days	Number of Cultures Not Showing CPE within 3 days but showing CPE within 30 days
2500	18	18	0	--
250	18	18	0	--
25	18	17	1	0
2.5	18	14	4	0
.25	18	13	5	0

NOTE: Cultures were infected with serially diluted virus and incubated at 37C. The number of cultures showing CPE within 3 days was determined and the TCD_{50} value was determined. Cultures not showing CPE within 3 days were incubated for an additional 27 days.

was done to determine if it were possible to either enhance the maintenance of the latent infection or to terminate the latent infection either with or without the development of a concomitant productive infection. Along with these experiments to determine the effects of the various chemicals on the latent infection, control studies were run to determine if these chemicals affected the productive infection at 37C.

The effect of hydrocortisone on the production of infectious HSV at 37C is shown in Table 8. Cultures were treated with 25 μg/ml of hydrocortisone and infected with approximately $5x10^2$ TCD_{50} of virus. After incubation at 37C for 2 days these cultures and control cultures not treated with the hormone were freeze-thawed 3X and assayed for virus in RK cells. Treatment of cultures with 25 μg/ml of hydrocortisone did not significantly affect the production of HSV at 37C. The hydrocortisone-treated cultures produced nearly as much infectious virus as control cultures. Furthermore, microscopic observation for CPE revealed as much cell destruction in the treated cultures as in control cultures.

The effects of two other steroid hormones, 17 B-estradiol and progesterone, on the production of HSV at 37C were also investigated. Table 8 shows that at concentrations of 2-5 μg/ml, 17 B-estradiol did not markedly affect production of HSV. Progesterone, however, did affect virus production at concentrations of 2-5 μg/ml. With 5 μg/ml of progesterone in the cell culture medium, the production of infectious HSV was reduced by greater than 99% as compared to untreated control cultures. CPE was markedly reduced in the treated cultures although

TABLE 8

GROWTH OF HSV AT 37C IN UNTREATED RK CELLS AND IN CELLS TREATED WITH VARIOUS HORMONES

Treatment		Amount of virus produced (TCD_{50}/ml)
None		3×10^5
25	µg/ml hydrocortisone	1×10^5
2	µg/ml 17 B-estradiol	5×10^5
5	µg/ml 17 B-estradiol	3×10^5
2	µg/ml progesterone	5×10^3
5	µg/ml progesterone	2.5×10^3
.01	µg/ml L-epinephrine	1×10^5
1.0	µg/ml L-epinephrine	5×10^5
2	µg/ml L-thyroxin	3.5×10^5

NOTE: Hormones were added to the culture medium 5 days prior to infection. A second treatment was given at the time of infection with virus. The same stocks of hydrocortisone, progesterone and 17 B-estradiol were used for both treatments. Fresh stocks of L-epinephrine and L-thyroxin were prepared for each treatment. Cultures were infected with 500 TCD_{50} of virus and incubated at 37C. After 48 hours duplicate cultures were freeze-thawed and assayed for virus in RK cells. Maximum titers in control cultures were reached at about 48 hours post infection.

focal areas of cell destruction were seen in these cultures. CPE spread slowly in these cultures and high titers of virus were never obtained. At 2 μg/ml of progesterone the inhibition of virus growth was almost as great as at 5 μg/ml.

Two other hormones, L-epinephrine and L-thyroxin were also investigated. In these studies L-epinephrine was added to the cell culture medium at concentrations of .01 μg/ml and 1 μg/ml. At these concentrations there was no significant effect on the production of infectious virus at 37C (Table 8). At a concentration of 2 μg/ml, L-thyroxin also had no effect on virus production at 37C.

To test the effects of the various hormones on the latent infection, cultures were treated with the various chemicals, infected with approximately 5×10^3 TCD_{50} of virus and incubated at 41C. Untreated cultures were infected with the same amount of virus and incubated along with the treated cultures. Cultures were transferred to 37C after 1-7 days and allowed to continue incubating at this temperature. After transfer to 37C cultures were monitored daily for CPE (Table 9).

Hydrocortisone treatment did not significantly affect the course of the latent infection. Virus was eventually recovered from 89% of the control cultures and from 85% of the cultures treated with 25 μg/ml of hydrocortisone. The average lag period of the hydrocortisone-treated cultures was slightly less than the average lag period of the control cultures. This slight difference was not statistically significant at the .05 level of confidence.

TABLE 9

RECOVERY OF HSV AT 37C AFTER INCUBATION AT 41C IN UNTREATED RK CELLS AND IN CELLS TREATED WITH VARIOUS HORMONES

Treatment	Number of cultures treated	Number of cultures in which virus was recovered after incubation at 37C					Average lag period (days)
		for 1-3 days	for 4-6 days	for more than 6 days	Total	(%)	
None	106	48	23	23	94	(89)	6.6
25 µg/ml hydrocortisone	40	19	11	4	34	(85)	4.7
2 µg/ml 17 B-estradiol	26	9	10	3	22	(85)	4.7
2 µg/ml progesterone	26	5	7	2	14	(54)	8.4
.01 µg/ml epinephrine	20	9	2	5	16	(80)	6.3
1.0 µg/ml epinephrine	20	10	1	8	19	(95)	7.3
2 µg/ml thyroxin	45	22	5	15	42	(93)	6.9

NOTE: Cultures were treated with the desired concentration of each hormone, infected with approximately 5×10^3 TCD_{50} of virus, and incubated at 41C along with untreated control cultures. Cultures were transferred to 37C after 1-7 days and continued incubating at 37C. Cultures were monitored daily for CPE and the lag period determined.

The results that were obtained when cultures were treated with 17 B-estradiol were similar to results obtained with hydrocortisone treatment. Virus was eventually recovered from 85% of the estradiol-treated cultures (Table 9). The average lag period of the treated cultures was slightly less than the average lag period of the untreated cultures but again this difference was also not statistically significant.

Treatment of cultures with 2 µg/ml of progesterone at the time of infection with virus and incubation at 41C did alter the course of the latent infection. Virus was recovered from only 54% of the progesterone-treated cultures as compared to 89% of the control cultures. This difference in recovery rates was significantly different at the .001 level. The average lag period of the progesterone-treated cultures was slightly longer than the average lag period of the control cultures, but this difference was not statistically significant.

When epinephrine was added to cultures that were infected with virus and incubated at 41C, there was no effect on the subsequent recovery rate of virus after these cultures were transferred to 37C (Table 9). Virus was eventually recovered from 19 of 20 cultures treated with 1 µg/ml of epinephrine and from 16 of 20 cultures treated with .01 µg/ml of epinephrine. Furthermore, there was no significant difference in the average lag periods of the treated groups as compared to the control groups.

Treatment of cultures at 41C with 2 µg/ml of L-thyroxin also had no effect on the rate of recovery of virus from these cells. Virus

was eventually recovered from 42 of 45 thyroxin-treated cultures or from 93% of the total (Table 9). Although this reactivation rate was slightly higher than the control rate, it was not significantly higher. The average lag period of the thyroxin-treated cultures was 6.9 days. This was also not significantly different from the control value.

Attempts at Modifying the Latent Infection by Treatment of Cultures with Metabolic Inhibitors and a Photosensitizing dye

In addition to these five hormones, three metabolic inhibitors, BUDR, IUDR and puromycin, and a photosensitizing dye, neutral red, were investigated in this model latent infection. The effects of these chemical agents on the productive infection at 37C were also investigated. Table 10 shows the marked inhibitory effects that BUDR had on the production of infectious HSV at 37C. There was a greater than 99% inhibition with 25 μg/ml of BUDR. At a concentration of 100 μg/ml there was a greater than 4 log reduction in virus growth. In these cultures, which were initially infected with about 500 TCD_{50} of virus, CPE could be seen. However, virus remained localized to very small focal areas. When cultures were treated with IUDR and infected with virus at 37C, the same inhibitory effects on virus multiplication were seen with this drug as were seen with BUDR (Table 10). There was a 99% inhibition of virus growth in cultures treated with 25 μg/ml of IUDR and a 3.5 log reduction in cultures treated with 100 μg/ml. At a concentration of 100 μg/ml, IUDR seemed to be more toxic to cells than was BUDR at

TABLE 10

GROWTH OF HSV AT 37C IN UNTREATED RK CELLS AND IN CELLS TREATED WITH BUDR AND IUDR

Treatment		Amount of virus produced (TCD_{50}/ml)
None		$5x10^5$
25	μg/ml BUDR	$5x10^2$
50	μg/ml BUDR	$1x10^1$
100	μg/ml BUDR	$1x10^0$
25	μg/ml IUDR	$5x10^3$
100	μg/ml IUDR	$1x10^2$

NOTE: Cultures were treated with BUDR and IUDR at the time of infection with virus. Cultures were infected with approximately 500 TCD_{50} of virus and incubated at 37C. After 48 hours duplicate cultures were freeze-thawed 3 times and assayed for virus in RK cells.

the same concentration. Many cells became granular and detached from the monolayer. The remaining cells did not undergo multiplication to replace the sloughed cells. Much less toxicity was noted at 25 μg/ml and so cultures were treated with this concentration in subsequent experiments.

Cultures were treated with 100 μg/ml of BUDR, infected with $5x10^3$ TCD_{50} of virus and incubated at 41C. Following incubation at 41C, the cultures were transferred to 37C. The BUDR-containing medium was replaced with inhibitor-free medium and the cells were incubated and monitored daily for CPE (Table 11). Although treatment of cells with BUDR did greatly suppress the growth of the virus at 37C, treatment of cells at the time of infection and incubation at 41C did not suppress the recovery of virus from these cells after transfer to 37C. Virus was recovered from these cells after transfer to 37C. Virus was recovered from 57 out of 80 treated cultures (71%) as compared to only 30 out of 50 control cultures (60%). Furthermore, virus was recovered within the first six days after transfer to 37C from 49 of the 57 BUDR-treated cultures as compared to only 17 of the 30 control cultures. This difference was statistically significant at the .025 level. This difference was reflected in the average lag periods of the treated and control groups. The treated group had an average lag period of 4.9 days as compared to 8.3 days for the control group. This difference was statistically significant at the .05 level.

TABLE 11

RECOVERY OF HSV AT 37C AFTER INCUBATION AT 41C IN UNTREATED RK CELLS AND IN CELLS TREATED WITH BUDR AT THE TIME OF INFECTION WITH VIRUS AND INCUBATION AT 41C

Treatment	Number of cultures treated	Number of cultures in which virus was recovered after incubation at 37C	Average lag period (days)
None	50	30 (60%)	8.3
100 μg/ml BUDR	80	57 (71%)	4.9

NOTE: Cultures were treated with 100 μg/ml of BUDR, infected with approximately 5×10^3 TCD_{50} of virus and incubated at 41C along with untreated control cultures. Cultures were transferred to 37C after 2-7 days. The BUDR-containing medium was removed from the cultures and replaced with inhibitor-free medium at the time of transfer of cultures from 41C to 37C. Cultures at 37C were monitored daily for CPE and the lag period determined.

The same experiment was run using IUDR instead of BUDR. Cultures were treated with 25 µg/ml of IUDR, infected with $5x10^3$ TCD_{50} of virus and incubated at 41C. The IUDR-containing medium was replaced with normal maintenance medium when the cultures were transferred to 37C. The results of this experiment are shown in Table 12. There was a slight reduction in the average lag period of the IUDR-treated cultures. This was similar to what was seen with the BUDR treatment. The overall reactivation rate of virus from the IUDR-treated cultures was greater than the reactivation rate of virus from control cultures. Virus was eventually recovered from 66% of the treated cultures and from 47% of the control cultures. This difference was statistically significant at the .01 level.

When BUDR or IUDR was added to cultures at the time of infection with virus there were no inhibitory effects on the eventual recovery of virus from these cultures. In contrast to this, it was found that when BUDR was added to cultures at the time of transfer from 41C to 37C, there was a very definite inhibitory effect (Table 13). Treatment of cultures for 4 days at 37C with 100 µg/ml of BUDR almost completely eliminated virus reactivations within the first 6 days. However, virus was not eliminated from the cells because subsequent virus reactivations did occur following removal of the inhibitor from the culture medium. The average lag period of the BUDR-treated cultures was over twice as long as the average lag period of the control cultures. This difference was significant at the .001 level of confidence. This difference was due to the reduction in the number of BUDR-treated cultures that had

TABLE 12

RECOVERY OF HSV AT 37C AFTER INCUBATION AT 41C IN UNTREATED RK CELLS AND IN CELLS TREATED WITH IUDR AT THE TIME OF INFECTION WITH VIRUS AND INCUBATION AT 41C

Treatment	Number of cultures treated	Number of cultures in which virus was recovered after incubation at 37C	Average lag period (days)
None	111	53 (47%)	9.0
25 μg/ml IUDR	109	72 (66%)	6.9

NOTE: Cultures were treated with 25 μg/ml of IUDR, infected with approximately 5×10^3 TCD_{50} of virus and incubated at 41C along with untreated control cultures. Cultures were transferred to 37C after 2-7 days. IUDR-containing medium was removed from the cultures and replaced with inhibitor-free medium at the time of transfer of cultures from 41C to 37C. Cultures at 37C were monitored daily for CPE and lag period determined.

TABLE 13

RECOVERY OF HSV AT 37C AFTER INCUBATION AT 41C IN UNTREATED RK CELLS AND IN CELLS TREATED WITH BUDR AT THE TIME OF TRANSFER OF CULTURES TO 37C

Treatment	Number of cultures treated	Number of cultures in which virus was recovered after incubation at 37C					Average lag period (days)
		for 1-3 days	for 4-6 days	for more than 6 days	Total	(%)	
None	32	14	4	7	25	(78)	5.4
100 µg/ml BUDR	32	2	0	15	17	(53)	11.5

NOTE: Cultures were infected with approximately 5×10^3 TCD_{50} of virus and incubated at 41C for 2-7 days. After incubation at 41C cultures were transferred to 37C. Half of the cultures were treated with 100 µg/ml of BUDR at this time. The BUDR-containing medium was replaced with inhibitor-free medium 4 days later. Cultures at 37C were monitored daily for CPE and the lag period determined.

lag periods of 6 days or less and to the increase in the number of cultures that had lag periods in excess of 6 days.

In another experiment cultures were treated with 100 μg/ml of BUDR at 2 or 5 days after transfer to 37C. By waiting before applying the treatment, it was possible to eliminate from the data all cultures in which virus growth occurred soon after transfer to 37C. By eliminating these early reactivations, the average lag periods of both the control group and the BUDR-treated group were increased (Table 14). The relationship between the two groups remained the same; the average lag period of the BUDR-treated cultures was over twice as long as the average lag period of the untreated cultures. This difference in the length of the average lag periods was significant at the .001 level. Finally, although the lag periods were increased by treatment of infected cultures with BUDR, the overall reactivation rate of virus from the BUDR-treated cultures was not affected.

Puromycin, a potent inhibitor of protein synthesis in mammalian cells (59) was tested for effects on the normal productive infection at 37C and for effects on the latent infection. This drug was much less inhibitory to virus growth at 37C than were the thymidine analogues, although there was some inhibition of virus growth at a concentration of 10 μg/ml (Table 15), a concentration which approached the toxic level. No significant reduction in virus growth was seen at levels of puromycin below 10 μg/ml and cell toxicity was observed at concentrations of 20-25 μg/ml. At these levels nearly all of the cells detached

TABLE 14

RECOVERY OF HSV AT 37C AFTER INCUBATION AT 41C IN UNTREATED RK CELLS AND IN CELLS TREATED WITH BUDR EITHER 2 OR 5 DAYS AFTER TRANSFER TO 37C

Treatment	Number of cultures treated	Number of cultures in which virus was recovered after incubation at 37C	Average lag period (days)
None	39	15	10.6
100 µg/ml BUDR	39	13	22.5

NOTE: Cultures were infected with approximately 5×10^3 TCD_{50} of virus and incubated at 41C for 2-7 days. After incubation at 41C cultures were transferred to 37C. After 2 days and after 5 days all cultures which had not yet shown CPE were divided into 2 groups. One group was treated with 100 µg/ml of BUDR. The other group served as a control group. The BUDR-containing medium was replaced with inhibitor-free medium after 4 days. Cultures were monitored daily for CPE and the lag period determined.

TABLE 15

GROWTH OF HSV AT 37C IN UNTREATED RK CELLS AND IN CELLS TREATED WITH PUROMYCIN

Treatment		Amount of virus produced (TCD_{50}/ml)
None		$1x10^6$
5	μg/ml Puromycin	$5x10^5$
10	μg/ml Puromycin	$5x10^4$

NOTE: Cultures were treated with Puromycin at the time of infection with virus. Cultures were infected with 1000 TCD_{50} of virus and incubated at 37C. After 48 hours duplicate cultures were freeze-thawed 3 times and assayed for virus in RK cells.

from the monolayer within 2-3 days.

Treatment of cultures with 15 μg/ml of puromycin at the time of infection with $5x10^3$ TCD_{50} of virus and incubation at 41C had 2 effects on the latent infection. Virus was subsequently recovered from a greater proportion of puromycin-treated cultures than from control cultures and the average lag period of the treated cultures was significantly longer than the average lag period of the control group (Table 16).

An attempt was made to eliminate virus from the latent infection by exposing infected cultures to neutral red, a photosensitizing dye, and then treating the cultures with strong visible light. Cultures were infected with $5x10^3$ TCD_{50} of virus and incubated at 41C. When the cultures were transferred to 37C, neutral red was added to the culture medium at a final dilution of 1:240,000-1:480,000, and the cultures were incubated in the dark for 2 hours. After 2 hours the dye was removed from the medium and the cells were washed in HBSS and exposed to strong visible light for an additional 2 hours. The cultures were then incubated at 37C in the dark along with control cultures and monitored for virus growth. The results of this treatment are shown in Table 17. The reactivation rate of virus from the photosensitized cultures was almost identical to the reactivation rate of virus from control cultures. Virus was recovered from 59% of both the neutral red-treated cultures and control cultures. Although the recovery rate was not altered by the photosensitization treatment, the average lag period of the treated cultures was significantly increased.

TABLE 16

RECOVERY OF HSV AT 37C AFTER INCUBATION AT 41C IN UNTREATED RK CELLS AND IN CELLS TREATED WITH PUROMYCIN AT THE TIME OF INFECTION WITH VIRUS AND INCUBATION AT 41C

Treatment	Number of cultures treated	Number of cultures in which virus was recovered after incubation at 37C					Average lag period (days)
		for 1-3 days	for 4-6 days	for more than 6 days	Total	(%)	
None	48	17	6	7	30	(63)	6.3
10 μg/ml Puromycin	45	11	7	22	40	(89)	11.8

NOTE: Cultures were treated with 10 μg/ml of Puromycin, infected with approximately 5×10^3 TCD_{50} of virus and incubated at 41C along with untreated control cultures. Cultures were transferred to 37C after 2-7 days. Puromycin-containing medium was removed from the cultures and replaced with inhibitor-free medium at the time of transfer of cultures from 41C to 37C. Cultures at 37C were monitored daily for CPE and the lag period determined.

TABLE 17

RECOVERY OF HSV AT 37C AFTER INCUBATION AT 41C IN UNTREATED RK CELLS AND IN CELLS TREATED WITH NEUTRAL RED AND EXPOSED TO STRONG VISIBLE LIGHT AFTER TRANSFER TO 37C

Treatment	Number of cultures treated	Number of cultures in which virus was recovered after incubation at 37C for 1-3 days	for 4-6 days	for more than 6 days	Total	(%)	Average lag period (days)
None	44	20	4	2	26	(59)	3.2
Neutral red	42	11	4	10	25	(59)	9.6

NOTE: Cultures were infected with approximately 5×10^3 TCD_{50} and incubated at 41C for 2-7 days. Cultures were transferred to 37C after incubation at 41C and divided into 2 groups. One group was treated with neutral red at a final dilution of 1:240,000. Cultures were treated in the dark for 2 hours. After 2 hours, the dye was removed from the cells, the cells were washed and fresh medium containing no dye was added to the cultures. Cultures were then exposed to strong visible light at a distance of 30 cm for an additional 2 hours. Following this, the treated cultures and control cultures were incubated at 37C and monitored daily for CPE.

Establishment of the Latent Infection in WI-38 Cells

One of the most important questions to be answered in this work was whether the latent infection was a characteristic feature of the virus in RK cells or whether a similar infection could be established in other cell types including cells of human origin. An attempt was made to establish the latent infection in WI-38 cells. The first step was to determine if virus growth was blocked at 41C in these cells. Cultures were infected with 2.5×10^3 PFU of virus and incubated at 41C. On the following 3 days duplicate cultures were freeze-thawed 3X and assayed for virus in RK cells. As seen in Figure 5, no virus was recovered from any culture. Microscopic observation of cultures incubated at 41C showed no detectable CPE on any day. In contrast, the virus grew very well in WI-38 cells at 37C (Figure 5). When cultures were infected with 1×10^3 PFU of virus, the cell cultures were completely destroyed in 2-3 days. The maximum amount of virus that was obtained from these cells was 7.5×10^6 PFU/ml. This titer was actually higher than had been obtained in RK cells.

Cultures of WI-38 cells were infected with between 5×10^2-1×10^4 PFU of virus and incubated at 41C for 1-6 days. Virus was able to survive in cells at 41C because after transfer to 37C, virus growth resumed in some of the cultures. Virus was recovered from 59 cultures out of the 121 cultures originally infected for a recovery rate of 49% (Table 18). Of these cultures 31% had lag periods of 4 or more days and 13% had

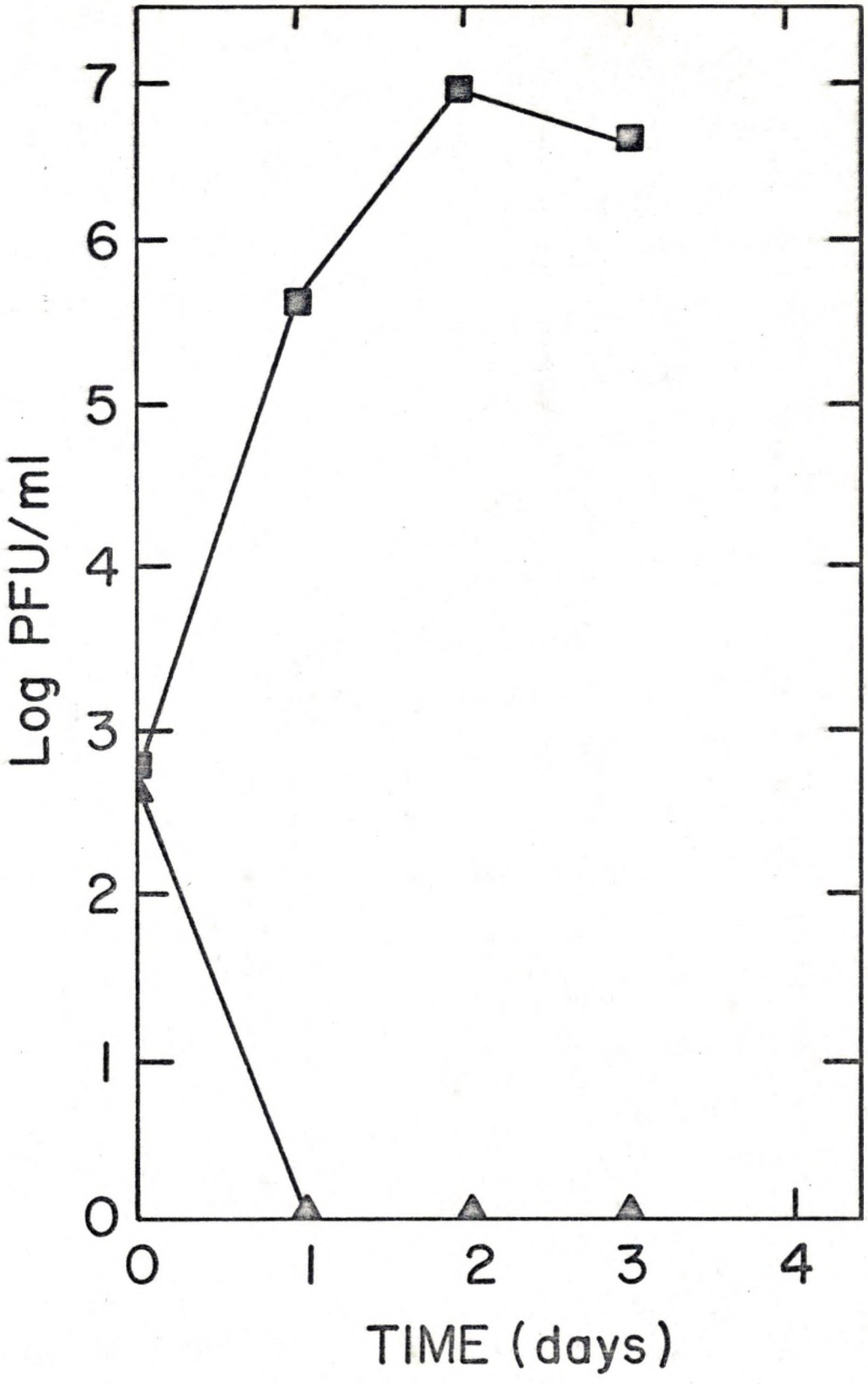

Log PFU/ml
0
1
2
3
4
5
6
7
0
1
2
3
4
TIME (days)

TABLE 18

RECOVERY OF HSV AT 37C AFTER INCUBATION AT 41C
IN WI-38 CELLS AND HK CELLS

Cell type	Number of cultures incubated at 41C	Number of cultures in which virus was recovered after incubation at 37C		
		for 1-3 days	for 4-6 days	for more than 6 days
WI-38	121	22	21	16
HK	62	4	4	0

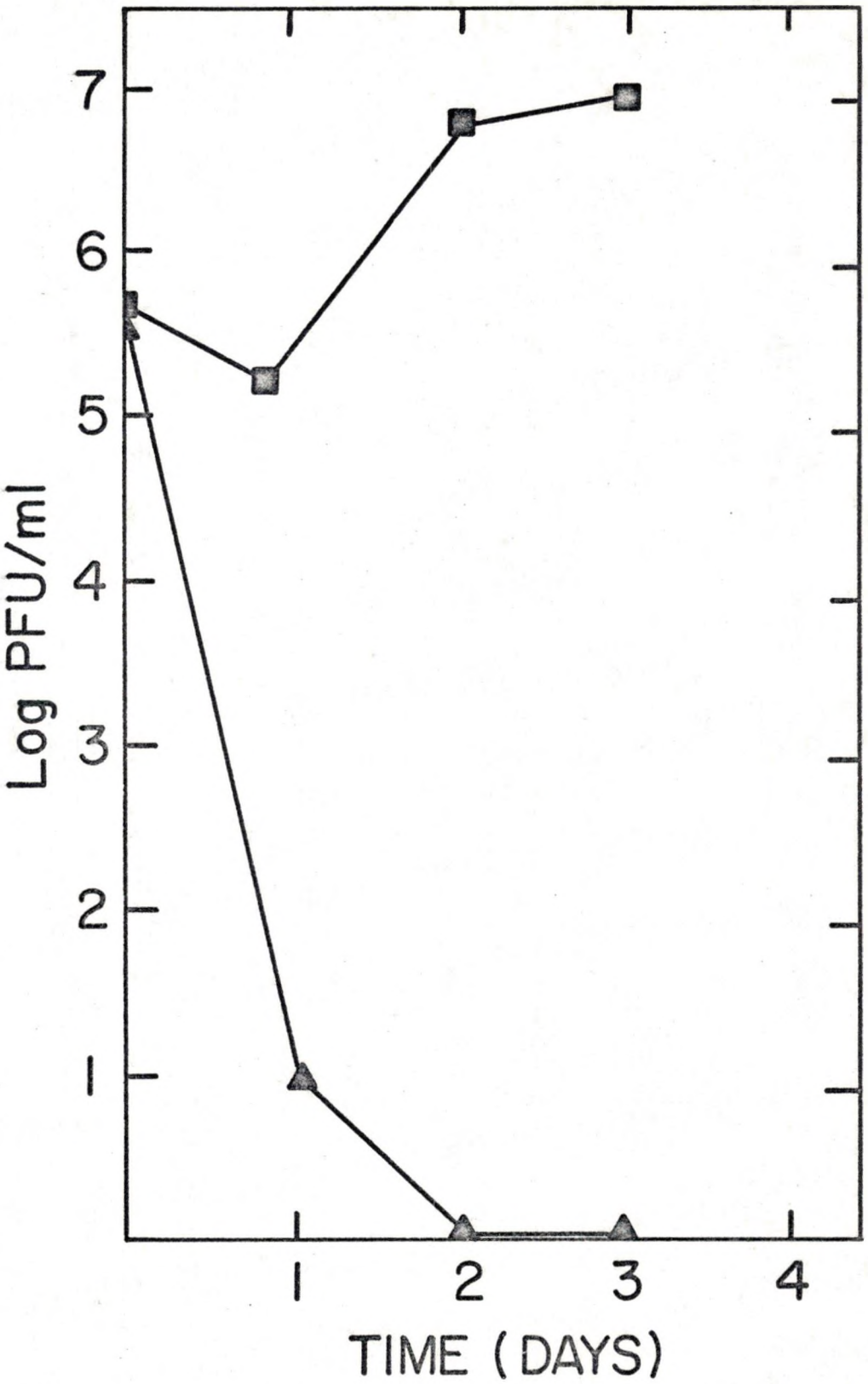
NOTE: Cultures were infected with $4x10^2$-$1x10^4$ PFU of virus and incubated at 41C for 2-7 days. After incubation at 41C cultures were transferred to 37C and continued incubating at that temperature. Cultures at 37C were monitored daily for CPE.

lag periods in excess of 6 days. The average lag period of this last group was 26.8 days and the longest lag period was 53 days. A latent infection similar to the one established in RK cells was established in WI-38 cells following incubation at 41C.

Failure to Establish the Latent Infection in Human Kidney Cells

The latent infection in WI-38 cells was qualitatively similar to the infection established in RK cells. Quantitative aspects were also comparable. An attempt was made to establish the latent infection in a third cell type, in human kidney (HK) cells. The inability of the virus to grow in HK cells at 41C is shown in Figure 6. When cultures were inoculated with a very high dose of virus, $5x10^5$ PFU/tube culture, and incubated at 41C for 1-3 days, only 10 PFU of infectious virus was recovered on day 1 and none were recovered on days 2 and 3. These cells were disrupted by freeze-thawing at the time of assay. The virus grew well in HK cells at 37C. High titers of virus were recovered on days 2 and 3 (Figure 6). At 37C cultures were entirely destroyed by the virus in 2-3 days while no CPE was seen at any time during incubation at 41C.

Attempts were made to recover virus from cells incubated at 41C by transferring viable cells to 37C and incubating them at this temperature. Cultures were originally infected with between $4x10^2$-$8x10^3$ PFU of virus/culture. The results are shown in Table 18. Virus was only recovered from 8 of 62 cultures (13%) after they were transferred from 41C to 37C. The cultures from which virus was recovered had all been


7
6
5
4
3
2
1
Log PFU/ml
1
2
3
4
TIME (DAYS)

incubated at 41C for 2-3 days. Virus was not recovered from any culture incubated at 41C for longer than this. Furthermore, the longest lag period observed in any of these 8 cultures was 5 days.

DISCUSSION

Two basic hypotheses have been proposed to account for the latent HSV infection at the cellular level. These two hypotheses are the dynamic state hypothesis and the static state hypothesis (26). The dynamic state hypothesis envisions a slow but constant replication of virus in the infected cells during the latency. Whole, infectious virus particles are always present but in very low numbers. According to the static state hypothesis, the virus is harbored in certain cells in which virus multiplication has been blocked. No infectious virus is present during the latency. It is not possible to state categorically that one or the other of the two hypotheses is correct. Certain evidence supports the dynamic state hypothesis while other evidence supports the static state hypothesis.

The results reported in this paper support the static state hypothesis. It is not likely that virus multiplication were occurring at a very slow rate in our model latent infection. It is also not likely that whole, infectious virus particles were present during the latent infection. Whole, infectious virus was found to be very labile at 41C. When virus was incubated at 41C in an environment devoid of viable cells, infectivity decreased at a logarithmic rate. The half-life of the virus under these conditions was only about 36 minutes. The virus was

stabilized slightly in the presence of viable RK cells, but inactivation was still very rapid. The half-life of the virus in viable RK cells was about 100 minutes. No virus was detected in cultures that were incubated at 41C for 1 or more days when the cells were disrupted at the time of assay. During the course of this study, a minimum of 50 cultures were examined for virus after freeze-thawing. An additional 40 cultures were also disrupted by sonication in an unsuccessful attempt to free virus which might be present. If virus multiplication were occurring during this period, or if whole, infectious virus particles were present, it should have been possible to isolate some virus from these cultures.

In addition to the fact that virus was never recovered from these cultures after incubation at 41C, it was also not possible to detect any CPE in the cultures incubated at 41C. If virus multiplication were occurring, although much less efficiently, it would be expected that some CPE would develop during this time as most of the virus would spread directly from cell to cell and form focal areas of infection. Furthermore, it is hard to rationalize the extended lag periods that were seen in a number of cultures after transfer to 37C with the idea that whole, infectious virus was present when these cultures were transferred to 37C. The cells were readily susceptible to infection with exogenous virus immediately after transfer to 37C.

It could be postulated that most of the inoculum virus was inactivated at 41C and that the lag periods observed in the cultures that

were transferred from 41C to 37C represented a normal delay associated with a very low concentration of virus. If this were true, it should have been possible to duplicate the extended lag periods by inoculating a very small amount of virus onto cells at 37C. The extended lag periods could not be duplicated in this manner. When virus was added to cells in graded amounts, virus growth always occurred rapidly with CPE being visible within 1-3 days or no evidence of virus growth was seen at all when cultures were incubated for up to 30 days.

Although whole, infectious virus was apparently not present in these cultures incubated at 41C, the virus was evidently not irreversibly inactivated because when the cultures were transferred from 41C to 37C and allowed to continue incubating at this temperature, virus was recovered from some cultures. In the control studies virus was recovered from 168 cultures out of 200 cultures of RK cells. Virus was also recovered from 59 out of 121 cultures of WI-38 cells. It was possible to show that some cultures contained many latent virus particles. When RK cells were infected with $1x10^4$ TCD_{50} of virus and incubated at 41C, as much as 1% of the inoculum virus was able to survive up to 6 days. In another study, a latent infection was established in the majority of the cultures that were infected with as few as 400 PFU of virus and in a few cultures that had been infected with only 40 PFU.

The results described in this paper concerning the development of virus-specific antigens in cells at 37C are in general agreement with the results of others (56, 60-64). However, differences from study to

study have been noted. These differences may, in part, be due to differences in the source and quality of the antiserum used, differences in the type of cells infected, differences in virus strains used and differences in staining technique. The antiserum used in this study was prepared in a manner identical to that used by Ross, Watson and Wildy (56), and the technique used in this study followed their technique. It is not surprising, therefore, that the results reported here are in agreement with their results. In their study fluorescence was detected in both the nucleus and the cytoplasm by 1-2 hours after infection of BHK-21 cells with HSV, and the infected cells were a mass of fluorescence by 9 hours post infection. In the study reported here, fluorescence was detected in both the nucleus and cytoplasm by 4 hours after infection. By 8 hours the cells were filled with an intense fluorescence. By this time many cells had rounded up and it was not possible to distinguish the nuclei from the cytoplasm of these cells.

These studies support the idea that the block in virus replication at 41C occurs at some point after completion of the initial events of the virus replication cycle. Fluorescence was seen in both the nucleus and cytoplasm of cells incubated at 41C for 1-4 days. Since fluorescence does not appear in cells infected at 37C until 1-4 hours after infection, it would seem likely that the events which occur during this initial period are also occurring in the cells incubated at 41C.

Although it could be shown that certain cells contained virus-specific antigens at 41C, it is not possible to say that viable virus was

preserved in these cells. No attempt was made to show that the fluorescing cells were, in fact, responsible for virus survival. To show that the fluorescing cells were responsible for virus survival, it would have been necessary not only to show a correlation between the presence of fluorescing cells at 41C and reactivation of virus upon transfer to 37C, but also to show a correlation between the lack of fluorescence at 41C and a lack of virus reactivations upon transfer to 37C.

Further work should be done on this aspect of the problem. Cells should be followed for a longer period of time at 41C to determine if the number of cells that fluoresce eventually falls to zero. It should also be determined if some cells begin fluorescing after transfer to 37C. Finally, and most importantly, it should be determined whether or not the cells that are fluorescing are responsible for the survival of virus.

Fluorescence microscopy of fluorochrome-stained cultures has proven to be a useful method for following the intracellular events that occur in virus-infected cells. AO is a particularly useful fluorochrome because under proper conditions of pH and ionic strength it differentially stains DNA and RNA (65). Under ultraviolet or near ultraviolet light, DNA has a green or green-yellow fluorescence while RNA has an orange or flame-red fluorescence. Armstrong, Hopper and Niven (66, 67) followed by AO staining the changes that occurred in the nuclei of adenovirus-infected hep-2 cells. They observed an abnormal distribution of chromatin within 24-48 hours after infection. In normal cells the fluorescing material was evenly distributed within the nucleus.

In the infected cells blocks of bright green-yellow fluorescence were seen scattered throughout the nuclei and especially around the periphery of the nuclei.

In the study reported here, changes in morphology of the nuclei of RK cells infected with HSV were observed. These changes resemble the changes seen after adenovirus infection. It is not surprising that similarities were noted because both HSV and adenovirus are DNA viruses that replicate in the nuclei of infected cells. In the normal, uninfected RK cells areas of bright fluorescence were commonly seen scattered throughout the nuclei. In the infected cells this brightly-fluorescing material was pushed to the periphery of the nuclei. These features could first be seen approximately 6-8 hours after infection. Prior to this the infected cells resembled the control cells.

None of the changes that were observed in cells infected at 37C were noted in cells that were infected with virus and incubated at 41C for 1-4 days. These cells appeared identical to control cells. Cultures incubated at 41C were infected with as much as $1x10^5$ PFU of virus/culture. Even if only 1% of this virus survived, it should have been possible to observe the characteristic cells because in control studies these changes could be seen when as few as $1x10^2$ cells were infected at 37C. Because no cells were seen with these features after incubation at 41C, it seems likely that the replication of virus was blocked at a time prior to when these changes occur.

The results of the fluorescent microscopy experiments support the idea that the virus replication cycle was blocked at a point following completion of the initial events but before gross morphologic changes occur in the cell. The exact part of the replication cycle that was blocked is not known. Other investigators have also found that replication of HSV-2 is inhibited at elevated temperatures (16, 17, 68, 69). Crouch and Rapp (70) reported that a type-2 strain of HSV was inhibited in hamster embryo cells at 39C. Transfer of cultures to 39C at any time during the first 6 hours of infection resulted in maximum inhibition, but transfer of cultures to 39C after 8 hours did not. It was found that viral DNA synthesis was greatly inhibited at 39C although some virus DNA synthesis did occur at this temperature, and the authors did not conclude that inhibition of viral DNA synthesis was solely responsible for the inhibition of virus growth.

It has been found that the thymidine kinase activity induced in cells by HSV-2 is very labile at 40C (15). Since this enzyme is involved in the synthesis of virus DNA, it is possible that the lack of virus growth at elevated temperatures is due to the inactivation of this enzyme.

The fact that virus replication was blocked at 41C does not necessarily imply that a latent infection was established. To fit the definition of a latent infection it is necessary that virus growth be blocked under conditions that are normally permissive to virus growth. CPE was seen in some cultures soon after transfer from 41C to 37C. In these cultures it is possible that virus growth was blocked at some point at

41C and continued from that point upon transfer to 37C. In other cultures CPE was not seen immediately upon transfer to 37C, but was seen after incubation at 37C for a longer period of time. It was in these cultures that the latent infection was established. It would be interesting to determine if there was a biologic difference between the cell-virus interactions in cultures which had no lag periods before CPE was seen and cultures which had extensive lag periods. It is possible that the virus genome was damaged in some cells at 41C and that the lag periods represented time needed for DNA repair. It is also possible that in the cells that had latent virus, the cells produced a repressor substance which inhibited virus growth after transfer to 37C. Although there is no evidence to support this idea in this model system, the development of a lysogenic infection in bacteria by specific bacteriophage depends upon the host cell repressing certain virus functions (71).

Certain features of this infection proved to be cell-dependent as differences were observed in the responses of 3 different cell types to infection with the virus at 41C. The 3 cell types investigated were RK cells, WI-38 cells and HK cells. The recovery rate of virus from the RK cells was 84%. Forty one percent of the cultures had lag periods of 4 or more days before virus growth was seen following transfer to 37C, and 24% of the cultures had lag periods of 7 or more days. The longest lag period observed was 45 days and the average lag period of the cultures with lag periods of 7 or more days was 15.3 days.

Qualitatively the response of WI-38 cells was similar to that of the RK cells although quantitative differences existed. The overall reactivation rate in WI-38 cells was 49%. Thirty one percent of these cultures had lag periods of 4 or more days and 13% had lag periods of 7 or more days. The longest lag period was 53 days and the average lag period was 26.8 days.

Although the recovery rate of virus from the WI-38 cells was significantly lower than the recovery rate from RK cells, the distribution of the reactivations was similar in both cell types. It is possible that at least some of the differences in the reactivation rates could be explained in terms of slight differences in the amount of virus originally added to the cultures. Experiments with each cell type were run separately. Some of the differences are likely to be cell-dependent differences. There are, no doubt, basic differences between the two cell types. RK cells are a primary cell type from New Zealand white rabbits and WI-38 cells are a limited passage cell line of human origin.

The response of HK cells to infection with the virus at 41C was very much different from the responses of RK and WI-38 cells. After transfer of HK cells from 41C to 37C the rate of recovery of virus was very low (13%) and no extended lag periods were observed. The reason for the low recovery rate from HK cells is not known. It is possible that virus was inactivated in the cells at 41C and that a latent infection was not established. On the other hand, it is possible that a latent infection was established in HK cells but that the cell-virus interaction was

very stable. It may be possible to determine between these two explanations. If HK cells do harbor the virus in a very stable latent infection, it may be possible to detect virus antigens in these cells. Of course, detecting antigens would not necessarily indicate that all of the virus genome is present or that it is undamaged and capable of replicating. Antigens have been observed in cells transformed by HSV, but the virus apparently never reactivates in these cells (33). If the virus is present in the HK cells and capable of growing, it may be possible to induce reactivations. The various methods that were used in this study in an attempt to increase the reactivation rate of virus from RK cells may be used.

The fact that cell-dependent differences exist in this latent infection indicate that this may be a useful model system in which to investigate the nature of host cell controlling factors in latent HSV infection.

Many factors have been associated with the periodic reactivations of latent HSV that occur in some of the people infected with this virus. Among the best documented stimuli of virus reactivation is trigeminal nerve surgery (20, 21). Other conditions reported to activate or predispose the individual to recurrent herpetic infections include various febrile diseases, particularly pneumococcal pneumonia and malaria, artificial fever therapy, menstruation, vaccine administration, emotional stress, trauma, sunburn and exposure to ultraviolet light, and certain allergic conditions such as systemic anaphylaxis and the localized

Arthus reaction (72-78). It has been proposed that certain of these stimuli may be mediated through "increased endocrine activity" (78). Three hormones, cortisone, epinephrine and thyroxin have been investigated in various model systems for effects on HSV infection. It was shown that treatment of experimental HSV-induced conjunctival ulcers in rabbits with cortisone aggrevated the infection process, resulting in more serious disease (79-82). In these experimental infections the virus was already actively growing, and it is likely that the treatment only made the tissue more susceptible to the virus. No attempt was made to activate a latent virus from infected cells. The well-known ability of cortisone to reduce non-specific inflammation and to cause involution of lymphatic tissues could account for the increased susceptibility of the treated tissues to the virus (83).

No reports were found in the literature relating thyroid hormone to virus reactivation, although excess thyroid hormone might be expected to influence virus reactivations because of its temperature-elevating effects (83). It has been reported, however, that cultures of hep-2 cells pretreated with 10 µg/ml of thyroid extract adsorbed HSV more readily than did non-treated control cells (84). While this might be of some consequence in an in vivo situation, it would not be expected to influence the growth of virus in cell cultures where the virus is left in contact with the cells continuously.

In another experimental situation, an encephalitis-like infection was established in young adult rabbits following an intracerebral

injection of HSV. Rabbits that recovered from the primary infection were treated with various chemical agents in an attempt to precipitate a second attack. Intramuscular injections of 2 mg of epinephrine precipitated a recurrence of virus growth in 6 of 10 animals. The infection in all 6 animals ended in a fatal encephalitis. While the epinephrine treatment did apparently lead to the recurrence of active infection, the virus was isolated from brain tissue from 6 of 8 animals which were not treated with epinephrine and which showed no evidence of virus growth. This would imply that the epinephrine did not cause a reactivation of a latent virus but only predisposed the treated animals to a serious infection with the virus that was already present (78).

In our studies treatment of RK cells with hydrocortisone, thyroxin, or epinephrine did not increase or decrease the susceptibility of the cells to HSV. When virus was inoculated onto cells at 37C, as much CPE was seen in cultures treated with each of the three hormones as in control cells and as much infectious virus was recovered from these cells as from control cells. When virus was inoculated onto hormone-treated cells that were then incubated at 41C, the reactivation rate of virus from the treated cells after incubation at 37C was similar to the reactivation rate from control cells. These studies indicate that the direct application of these agents to infected cells in culture was not effective in activating virus from what is apparently a true latent infection. However, since the relationship between this <u>in vitro</u> latency and the naturally-occurring <u>in vivo</u> latency is not known, it is not possible

to conclude that these hormones do not, in fact, contribute to the reactivation of virus in vivo.

Although neither estrogens or progesterone have been directly implicated in affecting latent HSV, it is possible that these hormones could have an effect on the latent infection. Certain clinical observations have indicated a correlation between the onset of menstruation in certain individuals and the development of cold sores (78), and experimental studies have indicated that mice are more susceptible to HSV during pregnancy (85). In our studies treatment of cells with 17 B-estradiol at 37C did not influence the susceptibility of cells to HSV. However, progesterone treatment inhibited the growth of the virus. When infected cultures were treated with 17 B-estradiol and incubated at 41C, no effect was observed on the subsequent reactivation rate after transfer to 37C. Progesterone treatment, however, significantly reduced the number of cultures from which virus was reactivated. The fact that treatment of cells with progesterone reduced the susceptibility of the cells to virus at 37C would account for the decreased reactivation rate after infection at 41C. The mechanism by which progesterone might induce increased resistance to the virus is not known. Morphologic changes were observed in the progesterone-treated cultures. It is possible that the number of receptor sites for HSV on the cell surface was reduced. It is also possible that this hormone, which has anti-anabolic properties, decreased some specific cell function which was necessary for maximum virus growth (83).

In a number of studies estrogenic compounds have been associated with neoplasms of the breast, vagina and cervix (86-90). In at least one experimental study, progesterone antagonized the tumor-promoting effects of the synthetic estrogenic compound, diethylstilbesterol (91, 92). If HSV-2 does have the potential to initiate oncogenic changes in cells, and if the oncogenic potential is enhanced by estrogenic compounds, cell culture models may be helpful in identifying this. Long-term studies may be necessary, however. In our studies the longest period of time that cultures were treated with 17 B-estradiol or progesterone was 14 days. Perhaps more dramatic effects would have been observed following long-term treatment with these hormones, either individually or in combination.

The antiviral effects of the pyrimidine analogues, BUDR and IUDR have been known for some time. Herrman was the first to report that IUDR was an effective inhibitor of the replication of several DNA-containing viruses, particularly vaccinia virus and HSV (93). Several reports have since confirmed that BUDR and IUDR inhibit HSV replication (94-98). Unlike fluorodeoxyuridine, BUDR and IUDR are apparently incorporated into viral DNA and inhibit virus growth after incorporation (99).

In our studies there was a very definite inhibition of virus growth in RK cells at 37C. Virus production was reduced by greater than 99% at concentrations of 25 μg/ml of either IUDR or BUDR and by 3-4 logs at concentrations of 100 μg/ml of either drug. The decrease

in virus production was paralleled by a decrease in the amount of CPE observed in the treated cultures.

Recent studies have indicated that these two pyrimidine analogues can stimulate active virus growth in cells that harbor virus in a latent state. Among the viruses reported to be activated by these chemicals are members of the oncornavirus group (100-107), papovaviruses (108, 109) and the Epstein-Barr Virus of the herpesvirus group (110-116). In addition to this, IUDR pretreatment of cells has been shown to enhance the replication of cytomegalovirus, another member of the herpesvirus group, perhaps by inhibiting some natural anti-viral mechanism of the cell (117, 118). The finding that latent viruses can be activated by treatment of the cells with these metabolic inhibitors has not been extended to HSV.

In our RK cell model no evidence was obtained that would indicate that HSV could be activated from a latent infection by treatment of the infected cells with BUDR. When cells were treated with BUDR either at the time of transfer from 41C to 37C or up to 5 days after transfer to 37C, the reactivation of virus in these cultures was transiently blocked. As a result of this transient block, the lag periods of many treated cultures was extended and the average lag periods of the treated groups were correspondingly longer than that of control groups. Although the reactivation of virus in BUDR-treated cultures was temporarily blocked, the overall reactivation rate of virus from these cultures was not significantly reduced.

It is possible that BUDR acted to prevent the reactivation of latent virus by some means. However, since BUDR was shown to be a potent inhibitor of virus growth in control studies, it is possible that the temporary block that occurred following BUDR treatment was due not to inhibition of virus reactivations but to the inhibition of active virus growth after virus reactivations occurred. In any event, the fact that the overall reactivation rate of virus in BUDR-treated cultures was similar to the reactivation rate in control cultures would indicate that BUDR treatment was not able to eliminate virus in the latent state.

The inhibitory effects that were seen when BUDR was added to RK cells at the time of transfer from 41C to 37C were not seen when the cultures were treated with the drug at the time of infection with virus and incubation at 41C. In fact, BUDR treatment at this time increased the reactivation rate of virus after transfer to 37C. In addition to this, the average lag period of the cultures treated with BUDR was shorter than the average lag period of the control cultures. Cultures treated with 25 μg/ml of IUDR at the time of infection with virus showed the same increased reactivation rate and the same shortened average lag period as were seen with BUDR treatment.

These findings with BUDR and IUDR indicate that treatment of cells under conditions where virus multiplication was already blocked tended to preserve virus and allowed for the more rapid reactivation of virus after the infected cultures were transferred to permissive conditions. Treatment of the cells with these potent metabolic inhibitors may

have acted to prevent the degradation of virus by the cells or may have blocked a specific step necessary for the formation of the latent infection. In any event, since these compounds are known to be potent inhibitors of virus growth after incorporation into the viral nucleic acid, these results indicate that virus multiplication at 41C was probably blocked prior to the synthesis of DNA. In light of these findings, more work should be done to determine if, in fact, treatment of cells after transfer to 37C can induce the reactivation of latent virus. It might be possible to induce reactivations without preventing active virus growth by giving cells a very short treatment (1-4 hours) or by cocultivating treated cells with non-treated cells.

Treatment of cells at 37C with puromycin resulted in a slight inhibition of virus growth. When cells were treated at 41C at the time of infection with virus, the average lag period of these cultures was subsequently lengthened after transfer to 37C. These effects could be due to the inhibitory effects of this antibiotic on cell metabolism. In addition to these results, the overall reactivation rate of virus from cultures treated with puromycin was greater than the reactivation rate of virus from control cultures. These findings may also be due to the inhibitory action of this drug. Perhaps less of the inoculum virus was able to be degraded in protein synthesis-suppressed cells.

Many viruses including HSV are known to be extremely labile in the presence of strong visible light after they have been pretreated with a light-adsorbing dye such as neutral red (119). The mechanism by

which inactivation occurs is referred to as photosensitization. Since photosensitization is now coming into widespread use as a treatment for recurring herpetic infections (120, 121), we felt that it would be worthwhile to see how this treatment affected the latent infection. Cultures were treated with neutral red dye and exposed to visible light at the time of transfer of the cultures from 41C to 37C. Treatment of cultures in this manner apparently did not affect the latent virus. Virus was recovered from the same proportion of treated cultures as from control cultures. Although the recovery rate was not altered by the photosensitization treatment, the average lag period of the treated cultures was significantly increased. Fewer reactivations were seen within 3 days after transfer to 37C in the group treated with neutral red. This inhibition could be due to inhibition of virus growth rather than inhibition of virus reactivations. Since photosensitizing dyes result in breaks in DNA after binding to the nucleic acid (122), it is possible that virus in the latent state is resistant to the binding by the dye.

SUMMARY

Replication of a type 2 strain of HSV was inhibited in RK cells when the cultures were incubated at 41C. Following transfer of the cultures to 37C after incubation at 41C, resumption of virus replication occurred in 84% of the cultures. However, in most of the cultures virus replication did not commence immediately after transfer to 37C. Forty one percent of the cultures had lag periods of 4 or more days and 24% of the cultures had lag periods of 7 or more days. During the period of time when no evidence of virus growth was seen at 37C the virus was latent.

The virus was maintained in some form other than as whole infectious virus particles at 41C because when the cells were disrupted by freeze-thawing or by sonication after incubation at 41C, no virus was recovered from any culture. Furthermore, rate of inactivation studies indicated that infectious virus disappeared very rapidly at 41C. The half-life of infectious virus at 41C in the presence of viable RK cells was only about 100 minutes. When viable cells were not present, the half-life of the virus was even less.

Two studies were done to quantitate the amount of virus that survived incubation at 41C in a form other than as whole infectious virus particles. From these studies it was estimated that 0.25-1% of the

inoculum virus was able to survive incubation at 41C.

Attempts were made to determine what stage of the virus replication cycle was blocked. Immunofluorescence studies indicated that virus-specific antigens were produced in cells infected with virus at 41C. However, it was not possible to say for sure that the same cells that contained virus-specific antigens also contained the virus that survived.

AO staining revealed that changes which occurred in the nuclei of cells infected with HSV at 37C did not occur in cells infected with the virus at 41C. Since these changes occurred in the cells at 37C 6-8 hours after infection, the block in virus replication at 41C most likely occurred at a stage prior to this.

Two experiments showed that although the virus failed to replicate in RK cells at 41C, preincubation of the cells at 41C did not prevent them from replicating exogenous virus after transfer to 37C. Virus attached to cells preincubated at 41C as readily as to cells preincubated at 37C, and cells preincubated at 41C produced as much infectious virus as cells preincubated at 37C.

An attempt was made to establish a latent infection by inoculating a very small concentration of virus onto cells at 37C. A latent infection could not be established in this manner. Growth of the virus occurred within 3 days or no growth occurred at all when the cultures were incubated for up to 30 days.

The ability to establish a latent infection was not a unique property of the virus in RK cells. A similar infection was established in WI-38 cells. An attempt to establish a latent infection in HK cells by the same method was unsuccessful, however. The basis for this cell-dependent difference is not known.

Cultures were treated with certain hormones and other chemical agents in an attempt to modify the latent infection. Hormones that were used include hydrocortisone, 17 B-estradiol, progesterone, l-thyroxin and L-epinephrine. Treatment of cultures with progesterone at the time that the cultures were infected with virus and incubated at 41C resulted in a significant reduction in the number of cultures from which virus was recovered after transfer to 37C. In control studies progesterone inhibited the production of infectious virus in cells at 37C. The other hormones tested did not have these effects. Treatments did not reduce the number of virus reactivations from cultures treated at 41C and did not alter the amount of virus produced in cultures at 37C.

Treatment of cultures with BUDR and IUDR significantly affected the latent infection. When the cultures were treated with BUDR at the time of transfer from 41C to 37C (after the latent infection had already been established) the average lag period was increased although treatment did not affect the overall recovery rate. When cultures were treated with BUDR or IUDR at the time of infection with virus and incubation at 41C, the subsequent lag period after transfer to 37C was actually reduced. In addition, with IUDR the overall recovery rate of virus from

the treated cultures was increased.

When cultures were treated with puromycin, the average lag period was increased. The overall rate of recovery of virus from the treated cultures was also increased.

Finally, treatment of cultures with neutral red and exposure of these cultures to strong visible light had no effect on the overall recovery rate but did increase the average lag period.

APPENDIX

The following is a list of terms which have been abbreviated in the text along with the corresponding abbreviations.

Herpes simplex virus	HSV
Herpes simplex virus type 1	HSV-1
Herpes simplex virus type 2	HSV-2
Rabbit kidney	RK
Hanks' balanced salt solution	HBSS
Minimal essential medium	MEM
Wistar-38	WI-38
Human kidney	HK
Cytopathogenic effect	CPE
Tissue culture infectious dose - 50%	TCD_{50}
Plaque forming units	PFU
Acridine orange	AO
Fluoresceinisothiocyanate	FITC
Phosphate buffered saline	PBS
5-bromo-2-deoxyuridine	BUDR
5-iodo-2-deoxyuridine	IUDR

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