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Studies on latent infection with Aujeszky's disease (Pseudorabies) virus

Edward Bryan Davies
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(PSEUDORABIES) VIRUS

Iowa State University

Ph.D. 1981

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Studies on latent infection with Aujeszky's disease (Pseudorabies) virus

by

Edward Bryan Davies

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Veterinary Microbiology and Preventive Medicine
Major: Veterinary Microbiology

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Signature was redacted for privacy.

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Ames, Iowa

1981
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INTRODUCTION AND REVIEW OF THE LITERATURE

The Ecology of Microorganisms

Microorganisms comprise a large and heterogeneous group of life forms which are found in almost all parts of this planet. Despite their small size, the combined weight of the microbial cells on earth is about twenty-five times greater than that of the planet's animal life (Dixon, 1979), and they contain, collectively, an enormous number of enzymes which are able to act upon a wide variety of substrates (Wiebe, 1971). The majority of microorganisms lead a saprophytic life, breaking down dead organic materials into simple constituents, and thereby returning them to the planet's circulating reservoir of nutrients (Odum, 1971; Ricklefs, 1976). Their large numbers, their wide range of enzymes, their ability to live in a wide variety of environments and to multiply or replicate rapidly when circumstances permit, make them ideally suited for those functions. A much smaller number of microorganisms have adopted a parasitic life-style, living inside the bodies of larger organisms. This adaptation has produced for the microorganisms a different set of problems than those encountered in the saprophytic environment, for the living host is not passive, but has active defense mechanisms which have evolved over the years to protect it against this invasion.

The ability of human beings and other animals to develop a resistance to further infection after a previous encounter with certain diseases must have been common knowledge for centuries. The scientific study of the phenomenon may, however, be said to have started with the
introduction into England from Turkey by Lady Mary Wortley Montagu around 1720 of the practice of variolization as a means of protection against smallpox (variola). It then received its great impetus with the discovery by Edward Jenner in 1796 that "matter" transferred from a cowpox lesion on the hand of Sarah Nelmes to the arm of James Phipps protected him against subsequent challenge with smallpox (Barrett, 1974). A substantial body of research has followed this pioneering work, and while much still remains to be learned of the precise mechanisms, it is now recognized that the higher animals have sophisticated defense systems using integrated cellular and humoral responses to clear their bodies of defective cells, invading microorganisms and foreign proteins, and that the efficiency of the processes is increased by a previous contact with those particular antigens.

Viruses are microorganisms which consist of a genome surrounded by a protective protein shell and sometimes by a protein and lipid envelope. They carry only a limited amount of genetic information. While many may live in a quiescence state for extended periods outside the living host, they are all obliged to live intracellularly in an organism at some time so that they may utilize their host's synthetic and energy-yielding apparatus in order to replicate (Martin, 1978). As they are therefore obligate parasites, it is essential for their survival that they have evolved in a manner which allows them to overcome their host's immune defenses. This they have done in a wide variety of ways. Some of these ways are discussed below, but the list is not proposed as definitive.

Some viruses simply circumvent the problem of host immunity. On
invading a susceptible animal, they replicate rapidly and their progeny are shed in large numbers. In due course, that animal develops resistance and clears itself of infection, and in all likelihood is completely refractory to a subsequent infection by the same virus. Meanwhile, some of the shed virions invade another susceptible animal and the process is repeated and continues while there is available a supply of animals which have not had previous contact with the virus. This requires a large host population, the size of which depends upon the life span and the rate of reproduction of those animals. In time, the adult part of the population will become resistant to the disease, and the virus will be dependent for its survival upon the birth of young susceptible animals. It is to the advantage of the virus not to kill the animals which it infects, so that although they may be refractory to subsequent infections, and so not of immediate value to the virus as a means of propagation, they serve as breeding stock, producing a new supply of susceptible animals. Fenner (1971a and b) has discussed the size of the human population needed to maintain a disease such as measles which does not usually kill its victims, which produces a solid life-long immunity, and which has no alternative hosts. In theory, 30 evenly spaced cases a year would be required to maintain the virus. In practice, about 3,000 cases are required, a level of occurrence which does not occur on isolated islands with populations of less than 300,000. As this virus has no alternative hosts which in effect would increase the population size, the virus has disappeared at intervals on all islands with populations of less than half a million. A shorter-lived, more rapidly breeding
animal could support such a virus with a lesser population size.

An alternative ploy is for the virus to change its antigenicity from time to time, so that it is not recognized by the host on a subsequent infection. This delays the host's immune response and clearance mechanisms and gives the virus the opportunity to multiply productively, rather than being destroyed before it can replicate. Such a technique is useful in long-lived, slow breeding hosts. Most adult human beings have had previous contact with influenza virus and the population as a whole is able to resist and stop the spread of the antigenic types of the virus which it has encountered before. Occasionally, however, there occur in the outer coats of the virions such substantial changes in antigenicity (Barry, 1980) that the virus is not recognized by the host, is treated as a primary infection with a relatively slow immunological response and clearance, and thereby the virus has time to replicate and spread to other persons before it is destroyed.

A third method by which a virus may circumvent host immunity is by in some way compromising that immunity. There are a number of viruses which do this and they use various not-yet-fully understood mechanisms. Lymphocytic choriomeningitis, if acquired congenitally by the mouse, persists for the whole of the animal's life and is shed continually in the urine, thereby acting as a source of infection for other mice. It is presumed that immune tolerance plays a part in the pathogenesis of the disease, but it is not complete, and circulating antibody-virion complexes may be found which may lead eventually to renal complications (Fenner et al., 1974). Other viruses have gone a step further, and have
become integrated into the host cell genomes, and have thereby acquired the ability to be passed from generation to generation of their hosts without provoking host immunological reactions (Fenner et al., 1974). Discussion of such viruses is beyond the scope of this work.

A fourth method of avoiding a population's immune defenses is used by the family of viruses which are the subject of this report. The classic example is the virus which causes chickenpox in human beings. Chickenpox is an acute infectious disease which spreads rapidly through a nonimmune human population and while doing so it immunizes that population, usually without serious effects. If this were the complete ecology of the disease, it would require, like measles as described earlier, a human population in excess of 500,000 in one geographical area to generate enough susceptible infants to maintain the virus. In practice, however, the virus is able to survive in much smaller populations. It does this by assuming a latent form in certain individuals after the primary acute infection, and it may then stay quiescent for a period of perhaps several decades, before recrudescing as shingles. This form of the disease produces cutaneous vesicles from which chickenpox virus is shed, and which may infect susceptible infants. The virus may, therefore, apparently disappear for years, but can then reappear when a new generation of susceptible hosts has been born. It can thereby maintain itself in populations of less than 1000 individuals (Fenner, 1971a and b).

The phenomenon of latency is of great medical importance. It is naturally of great concern to the individuals which suffer recurrence of a disease acquired years ago, and it is also of great concern to those
responsible for control, and perhaps eradication, of diseases in the population as a whole. The work described in this dissertation investigates certain aspects of the phenomenon.

**Herpes simplex** Type 1 in Human Beings and Laboratory Animals

In the human being, the most common effect of this virus is the production on the lips of the familiar "cold sores" or "fever blisters". These are unsightly and inconvenient, but are of little consequence. Unfortunately, however, the virus may cause lesions on the cornea, leading to serious scarring, and encephalitis (Price et al., 1975; Wheeler, 1975). These dangerous manifestations have led to considerable interest in this virus and this, together with the early development of small animal models, has resulted in the virus being the best-studied of the herpetoviridae and a model for related viruses. In this section, the literature is reviewed so as to provide a background for the subsequent sections.

The primary infection of the human patient is usually not noticed. If it causes systemic effects, these are not characteristic and are not likely to be specifically identified. Burnet and Lush (1939) and Scott (1957) believed that there is an early period of susceptibility in the child after loss of maternal antibody and that the child is particularly vulnerable in the preschool years. This view was supported by Cesario et al. (1969), who noted fewer sero-conversions in older children, possibly because the children while at play had less intimate contact with each other than younger children. Older persons who have not had
previous contact with the virus are not, however, refractory. Stern et al. (1959) documented the infection of susceptible adult nursing staff members.

The incidence of *Herpes simplex* type 1 in the human population is very high. Burnet and Lush (1939) found antibodies in 51 of 55 (92.7%) of adult hospital patients tested, and Cesario et al. (1969) found antibodies in 73 of 143 (51%) of healthy children tested.

Clinically healthy people are frequently found to be excreting virus. Cesario et al. (1969) found virus was being shed into the pharyngeal secretions of 8-10% of children aged 3-14 years at any one time, and that the virus was shed at least once by about one-fourth of the children in their study in the period of a year. Similarly, Douglas and Couch (1970) were able to isolate virus from 3.6% of oral secretions collected from healthy adults.

When viral shedding recrudesces in the human patient, it may cause the formation of vesicular lesions, and it is common knowledge that these tend to recur on the same site, often a very narrowly-defined portion of the lip. It is also well-known that these recrudescences may occur periodically for many years. Documentary evidence for this has been provided by the observation that herpetic whitlows induced on the fingers of nurses during contact with infected patients at recorded times may recur at exactly the same site for up to 10 years at least (Stern et al., 1959). Herpetic lesions induced on the thigh or abdomen of patients with infectious material derived from their own penile lesions in an attempt to induce enhanced immunity, have reappeared at the inoculated
sites (Lazar, 1956; Blank and Haines, 1973).

These recrudescences may be provoked by a very large number of disparate naturally-occurring stimuli, such as the common cold (hence "cold sore"), fever (hence "fever blister"), menstruation, administration of vaccine, emotional stress, trauma, severe sunburn, allergic reactions, chemical irritants, secondary dentition, coitus and digestive disturbances (Schmidt and Rasmussen, 1960).

Recrudescences may also be provoked in the human patient by artificial stimuli. Some years ago it was the vogue to treat certain clinical conditions by artificially elevating the patient's temperature. It was noted in one study that when this was done Herpes simplex virus type 1 vesicles recrudesced in 46.2% of the patients (Warren et al., 1940), a high figure which serves as additional evidence of the pervasiveness of this infection in the human being. Similar results were obtained by Keddie et al. in 1941. In addition, it has been recorded that active infection may recrudesce in the human patient after a renal transplant (Montgomerie et al., 1969), but which of all the various factors involved in such a procedure, including surgical shock and immunosupression, has caused the recrudescence is not known. The recrudescence of active Herpes simplex virus infections after surgical manipulation of the trigeminal ganglion will be discussed in a later section.

Recrudescence of active infection has also been noted in laboratory animals which had been artificially infected with the virus some time earlier. In the rabbit, Good and Campbell (1945, 1948) noted that anaphylaxis provoked a recrudescence which manifested itself as a herpetic
encephalitis, and Schmidt and Rasmussen (1960) obtained a similar effect after the injection of synthetic adrenalin, but in neither of these procedures was virus actually shed from the animal. In 1961, however, Anderson et al. used the Arthus reaction to provoke recrudescence and shedding of virus in the eye of the rabbit, and in 1966, Laibson and Kibrick obtained a similar effect using epinephrine in the rabbit's eye. This latter observation was of some clinical importance, as the drug has been used in human beings for the treatment of glaucoma.

In the mouse, Stevens et al. (1975) were able to provoke recrudescence of the virus in nervous tissues without observable shedding to the exterior by the production of pneumococcal pneumonia. Robinson and Hurd (1975), however, were able to produce recrudescence of skin lesions by treatment with cyclophosphamide and prednisolone sodium phosphate. In another laboratory, workers were able to produce recrudescence in the mouse ear by mild trauma (Hill et al., 1978) or by stressing with ultraviolet light or by injections of prostaglandin E₂ (Blyth et al., 1976).

Despite this formidable list of stimuli which have been documented as provoking recrudescence of the virus, it should not be assumed that a recrudescence only occurs after some well-recognized stimulus. Most human sufferers from labial herpes are aware that sometimes recrudescences occur without their being able to identify a provoking stimulus. This phenomenon has been documented by Douglas and Couch (1970). A similar situation has been demonstrated in the rabbit, in which spontaneous recrudescence with shedding of virus from the cornea has been noted in the absence of any known provocation from periods of up to 24 months.
after initial infection (Anderson et al., 1961; Nesburn et al., 1967) in the mouse, in which there may be recrudescence causing erythema of the ear for up to 160 days after clinical infection (Hill et al., 1975), and in the guinea pig, in which there may be recrudescence causing inflammation and often vesicles and haemorrhagic macules on the footpads for up to 1½ years after initial infection (Scriba, 1975).

In view of these spontaneous recrudescences in the absence of known stimuli, care should be taken when attributing recrudescence to suspected stimuli. The apparent relationship may be only coincidental.

It has been known for a considerable time that the distribution of recurrent herpetic lesions is related in some way to the distribution of the peripheral nerves. Perhaps the first to note this was Mehlis in 1818 who wrote "that a herpetic eruption follows the distribution of peripheral nerves" and he was supported in this by von Barënsprung who in 1863 wrote "that it was a manifestation of lesions of the posterior root ganglion", both these authors being cited by Cushing (1904a). Whether these authors were referring to Herpes simplex type 1 or 2, or to another of the zosteriform hepetoviridae, Varicella/Zoster, we cannot say at this time.

Direct and unequivocal evidence of the relationship between Herpes simplex virus and nervous tissue infection was provided by Cushing (1904a, 1904b, 1905), when he reported that facial herpes was induced in patients after surgical incision of the trigeminal ganglion to relieve unbearable and otherwise incurable facial neuralgia. This phenomenon has been confirmed many times since, as by Carton and Kilbourne (1952), and Carton (1953), who showed that recrudescence may occur in
up to 93% of persons so treated, a figure which serves to emphasize again the universality of infection by this virus. These authors also pointed out that if patients were subjected to repeated surgery, recrudescence after the second manipulation of the trigeminal ganglion did not occur in skin denervated during previous surgery.

Other supporting but not unequivocal evidence of the relationship of herpes and nervous tissue infection is afforded by the common observation that facial herpes is often preceded by a characteristic tingling sensation (Baringer, 1974), and by observations that intracranial neuralgia may in some patients regularly precede facial herpes (Behrman and Knight, 1954; Constantine et al., 1968).

Experimental investigation of this phenomenon became possible after the work of Löwenstein (1919) and Grüter (1920), who, as cited by Paine (1964), showed that rabbits could be infected by the agent which caused herpetic lesions in humans if it was introduced onto their scarified corneas. This discovery allowed experimentation from which an explanation of the phenomenon of the relationship of the virus with infections of nerves and its periodic recrudescence was developed (review by Goodpasture, 1929).

In their experiments, Goodpasture and Teague (1923) inoculated rabbits at various sites and then observed their tissues after various periods for inflammatory changes and intranuclear inclusion bodies. In some cases, they accentuated the lesions by injecting trypan blue before death (McClellan and Goodpasture, 1923).

They showed that virus was transmitted along sensory nerves
following inoculation on the rabbit cornea, and that in due course it caused unilateral lesions in the pons and medulla oblongata; secondly, that it was transmitted along motor nerves following inoculation into the masseter muscle, and that it again caused lesions in the pons and medulla oblongata. They also showed that it was transmitted along sympathetic nerves following inoculation of virus into the ovary and that it caused myelitis of midthoracic segments of the spinal cord and paralysis of the caudal half of the body. They emphasized the dependence of the virus on neural spread following inoculation of an appropriate muscle with and without severance of the sciatric nerve, noting that severance prevented myelitis of the spinal cord.

In addition to these histopathological observations, they demonstrated severe clinical manifestations in guinea pigs in which the skin was first sensitized with coal tar, and then injected with *Herpes simplex* to give a local lesion; this was followed a few days later by a more generalized but still zonal herpetic lesion. This indicated that virus had spread centripetally from the initial site of cutaneous infection to the central nervous system, had spread there, and had then returned to a larger area of skin by centrifugal spread down the nerves (Teague and Goodpasture, 1923).

There was little that could be added to this definitive set of experiments until new techniques became available. Johnson (1964), using immunofluorescent antibody techniques, believed that viral spread occurred in the endoneural cells (Schwann cells and fibroblasts) rather than in the axons. Cook and Stevens (1973), however, in their review of previous
work and discussion of their own results, were not convinced of this, and believed that Herpes simplex virus could travel in the axons themselves. Although the issue remains unresolved today, it is under intense study, and whatever the eventual outcome of the debate will be, all are presently agreed that Herpes simplex virus can travel centripetally from the epithelium to the central nervous system.

In addition to spread along the nerves, virus may also reach the nervous tissue and become latent there via the bloodstream, as was shown by Cook and Stevens (1976) in mice inoculated intravenously. Interestingly, the virus appeared to have a predilection for nervous tissue, and could not be found subsequently in other tissues.

In 1929, Goodpasture delivered the De Lamar Lecture before the Johns Hopkins University School of Hygiene and Public Health, and in it he reviewed the state of knowledge of herpetic infection and its relationship with the nervous system. It is worth quoting parts of this historic lecture in his own words.

The frequent outbreaks of herpetic eruptions in the course of certain infectious diseases, and especially in association with intoxications of various kinds, render it very probable that the virus may reside in a latent form indefinitely in a large proportion of a selected population. The recurrence of herpetic eruptions breaking out in certain individuals at practically the same site time and again, are an evidence of a latency of the virus in intereruptive periods.

He then commented that certain authors were unable to find virus in skin after the lesions healed, and went on to say,

we do not know where the virus resides if it remains latent in intereruptive periods. The results of animal experimentation have given us, however, a rational basis for suspecting that it may be harbored in an inactive state within the nervous tissues, particularly the nerves and ganglia.
This hypothesis has been under debate ever since.

Support for the hypothesis came from the results of the artificial fever therapy already described, in which induced fever provoked recrudescences of cutaneous herpes (Warren et al., 1940; Keddie et al., 1941). It was also supported in a negative way by the failure of many workers to find virus in the skin between herpetic episodes (review by Goodpasture, 1929), and by the inability of workers to transfer a site of recurrence of lesions by switching skin grafts in the same patient. This last approach, often quoted, and taken to be strong evidence of reinfection of the skin by centrifugal spread from the nerves rather than by recrudescence in the skin itself, is not in the English literature, and appears to have been done with only 2 or 3 patients, according to the review by Wheeler (1975). It should therefore not be taken as definitive.

More recently, however, and with access to modern virological techniques, Rustigian et al. (1966) were unable to find virus in skin or oral mucosa between episodes.

The biggest objection to Goodpasture's hypothesis, and it was a serious one, was the failure of a series of workers to isolate the virus from excised trigeminal ganglia.

Burnet and Lush tried and failed in 1939. In their report, they were, however, cautious enough to say "it is conceivable that dormant virus in a cell may not be liberated by grinding or even by digestion of the cell in the tissues of the inoculated rabbit."

The failures of subsequent workers to isolate virus from the human trigeminal ganglion led to their proposing alternative hypotheses for the
appearance of facial herpes after surgery on the trigeminal ganglion.

Richter (1944) suggested that "a more likely explanation of postoperative herpes would appear to be an alteration of the metabolic state of the (denervated) skin such as would predispose it to invasion by external virus."

Carton and Kilbourne (1952) took this idea further, and suggested "that virus is activated in situ as a result of local changes in tissue metabolism secondary to interference with the sensory innervation of latently infected tissue", and Carton (1953) used the theory to explain why herpetic lesions did not appear in previously denervated areas of the skin after interference with the trigeminal ganglion by suggesting that an intact pathway was necessary for the irritative phenomenon to be transferred from the ganglion to the end organ. Kaufman et al. (1968) were even less convinced of the need to postulate latent infection in nervous tissue. When discussing recurrences of herpetic keratitis, they suggested that chronic infection of the conjunctiva and lacrimal glands could provide a source of virus for recurrent infection.

The work in human beings was now at an impasse, and it was left to workers with laboratory animal models to make further progress. Early investigators had used rabbits and guinea pigs to identify herpetic infections as being infectious in nature and to show the spread of the infection in nerves (reviewed by Goodpasture, 1929). This work had, however, not shown that the early acute infection was followed by a latent infection. Good and Campbell (1945), however, were able to show that rabbits could maintain a latent infection for at least 6 months and that
anaphylactic shock could provoke an encephalitic recrudescence. They expanded their work (Good, 1947; Good and Campbell, 1948) to show that the latent infection could be maintained for at least 9 months and that recrudescence could be induced by electric shock, and Schmidt and Rasmussen (1960) showed that epinephrine also could act as an inciting agent. Walz et al. (1974) were able to induce recrudescence of virus in the neural ganglia in mice infected by a variety of routes by cutting the appropriate nerves. Anderson et al. (1961) were able to provoke shedding of virus to the exterior, rather than just herpetic encephalitis, by using the Arthus reaction in the rabbit's eye; Laibson and Kibrick (1966) provoked herpetic keratitis in the rabbit by intramuscular injection of epinephrine; Walz et al. (1974) provoked cutaneous recrudescence in mice by cutting nerves; and in 1977, Nesburn et al. were able to simulate Cushing's work by reactivating Herpes simplex virus with ocular shedding by surgical probing of the trigeminal ganglion in the rabbit.

Meanwhile, the spontaneous shedding of virus had been noted in the rabbit's eye by Anderson et al. (1961) and Nesburn et al. (1967), and for periods of up to 1½ years from the footpads of guinea pigs (Scriba, 1975).

The efficiency of the establishment of latency was demonstrated by McKendall et al. (1974) when they showed that it only required \(10^2\) TCID\(_{50}\) inoculated into the mouse footpad to establish the infection.

This multitude of models in various animal species suggested that there was a universality to the phenomenon of herpes virus latency, and
together with the experiments of Stevens and Cook (1971), opened up the field to further investigation.

Stevens and Cook (1971) described their experiments to induce latency in mice. They inoculated the mice in the rear footpads. About 80% of them became paralyzed, and about half of these recovered during the next several weeks. These recovered mice were killed at intervals from 2 weeks to 3 months after the initial infection and their spinal ganglia were ground up and tested for infectious virus. None was found. Other samples of the spinal ganglia were, however, not ground up, but were instead maintained as organ cultures in nutrient medium, and when the supernatant fluids were tested on the 7th and 14th days, virus was frequently found. The inference of this important discovery was that in a latent infection, the virus was in what these workers called a "sub-viral state" in the ganglionic neurons or supporting cells, and this provided a possible explanation for the failure of earlier workers to recover virus from the trigeminal ganglia of human cadavers.

In a subsequent paper, Stevens et al. (1972) showed that their organ culture technique could be used to recover latent infection from the trigeminal ganglia of rabbits which they had infected in the eye 4 to 8 months previously. In this paper, the authors commented that they did not know how the viral genome was maintained in the ganglia, and suggested that as the morphological techniques and the assay methods for infectious virus seemed to be insensitive, they could not exclude the possibility that there was continued replication of a small amount of infectious virus.
The persistence of Herpes simplex virus in various parts of the nervous system of a variety of animal species and the ability of the organ culture technique to recover it were confirmed by a number of workers over the next years. From rabbits, Benda et al. (1973) were able to recover virus for up to 4 months from the trigeminal ganglia after infection of the dental pulp or cornea, and Knotts et al. (1973), Baringer and Swoveland (1974) and Martin et al. (1977) were able to recover virus for up to 12, 11 and 6 months, respectively, from the trigeminal ganglia after infection of the eye. From mice, Price et al. (1975) were able to recover virus for up to 14 months from the superior cervical autonomic ganglia after infection of the eye. While it is not a direct part of this discussion, Reeves et al. (1976) were able to show that the related virus, Herpes simplex virus type 2, could be recovered in a similar latent manner from the trigeminal and sacral root ganglia of experimentally infected Cebus monkeys.

The success of Stevens and Cook (1971) and of these other workers in obtaining recrudescence of virus from excised tissue fragments using organ culture had, of course, been noted by the human pathologists, and it was natural that they should use the technique to search for virus in the human trigeminal ganglion.

Bastian et al. (1972) recovered Herpes simplex virus from the trigeminal ganglion of 1 of 22 patients at autopsy by co-cultivating fragments of the tissue with Vero green monkey cells. Free infectious virus capable of causing cytopathic effect in the Vero cells was not released until after 3 weeks of culture.
This work was quickly confirmed by other workers. Baringer and Swoveland (1973) recovered Herpes simplex virus from the trigeminal ganglia of 6 of 7 patients at autopsy using a slightly different method. They simply maintained the fragments of tissue in nutrient medium and looked for cytopathic effect in the cells which subsequently grew out from the fragments. The cytopathic effect occurred in 10-45 days after explantation. Rodda et al. (1973) used a similar technique on fragments of the trigeminal ganglion derived from 1 patient, and they were able to observe cytopathic effect in the tissue outgrowths in 4 weeks. Plummer (1973) trypsinized human trigeminal ganglia and inoculated the resulting cell clumps into cultures of primary rabbit kidney cells, and was able to observe cytopathic effect in from 7 to 17 days in 4 of 10 tests.

The generality of the system was shown by Baringer (1974) who, using a co-culture technique with human embryonic lungs, was able to obtain Herpes simplex virus type 1 from the trigeminal ganglion, and Herpes simplex virus type 2 from the sacral ganglion of the same patient, consonant with the known propensity of the 2 viruses to cause oral and genital infections, respectively.

The Mechanism of Latency of Herpes simplex Type 1

When a person who has had epithelial lesions associated with Herpes simplex virus type 1 infection develops another lesion on the same site, there is no clinical means of knowing if this lesion is a recrudescence of latent infection or a reinfection from an exogenous source. The mere presence of circulating antibodies in the patient at the time of the latest
infection does not preclude exogenous infection. As Blank and Haines (1973) pointed out, previously-infected human beings with circulating antibodies may easily be reinfected under controlled conditions.

Roizman (1974), however, stated the situation quite succinctly, and it is appropriate to quote him.

The fact that recurrences are predictable strongly suggests that they are not the result of reinfection with virus from sources exogenous to the body but rather that the virus causing the recrudescence resides in the body in the interim between recrudescences.

This view is now generally accepted. It immediately raises the interesting question, again posed by Roizman, as to how can a virus which is invariably lethal to cells yet be latent in the host?

Much work has been invested in this problem, but as yet there are no clear answers. The subject has been reviewed by many authors, particularly Paine (1964), Docherty and Chopan (1974), Roizman (1974) and Wheeler (1975).

Stevens et al. (1975), proposed what they called a "general hypothesis", in which they said

recurrent herpetic disease involves initial infection and replication of Herpes simplex virus in the skin, mucus membrane or eye, with subsequent centripetal passage in sensory nerves to corresponding ganglia where a latent infection is established. After "reactivation" by one of many diverse stimuli, the virus would pass centrifugally in the sensory nerve, ultimately reaching the surface where lesions would again be produced.

To establish the validity of the hypothesis, three phenomena would have to have been shown.

First, and most importantly, after infection of tissues at the body surface, virus must be shown to reach and then be selectively harbored in sensory ganglia. Second, the
infection must be demonstrated to travel to and from the ganglia in corresponding nerve trunks. Finally, a defined manipulation of the experimental animal must be followed by reactivation of virus and subsequent reappearance of lesions on the external surface.

The first two of the requirements have been shown quite convincingly, but the third has not. The recrudescences in the skin which have been shown following stimulation of the trigeminal ganglion could have been from virus in the ganglion, already in the skin, or from an exogenous source.

Some workers believe that the latent virus is resident in the skin. This hypothesis has the advantage of being the simplest. It was supported by Burnet in 1955, who pointed out that a persistent replication of virus could occur in the lower rapidly-multiplying layers of the skin epithelium with no major damage to the host. Attempts to find virus in the human skin between recrudescences have been unsuccessful, but as it took many years to find virus in the trigeminal ganglion, it cannot categorically be said that it does not exist. Scriba (1977) was able to find virus in the skin of the footpads of 6 of 8 guinea pigs tested, but not in the nervous tissue, for up to 313 days after infection. Unfortunately, it is not certain that the virus had been resident in the skin for the whole of that period. Conceivably, it could have just been shed there, as in Hill and Blyth's skin trigger hypothesis of 1976. Experiments using neurectomized guinea pigs may in due course give the answer.

Support for the idea that latent Herpes simplex virus type 1 is resident in the skin came from Sabin (1975). He claimed that local treatment of cutaneous lesions with ether did, by destruction of the virus,
prevent reinfection of the local cells and in due course eliminated the recrudescence at that site. Even if this form of treatment should prove broadly applicable, it would not prove that recrudescence depends upon local virus rather than upon virus derived from the nervous tissue. As Wohlenberg et al. (1976) pointed out, recurrent cutaneous episodes might lead to subsequent infection of otherwise uninfected nerve cells, thereby perpetuating the ganglionic infection. Local treatment with ether could merely interrupt this cycle.

Despite the work of Sabin (1975) and Scriba (1977), most workers today accept the hypothesis that the latency occurs in the neural ganglion. Time will tell whether this faith is justified.

There are two basic alternative hypotheses of mechanisms of latency (Roizman, 1966). These hypotheses propose:

1) Dynamic State Hypothesis. The virus is constantly multiplying in chronically infected tissue at the same or a distant site from the site of recrudescence, and certain stimuli "heighten" the susceptibility of the tissues in some way.

2) Static State Hypothesis. The viral genome is conserved in a nonproductively infected cell and the infection becomes productive only when the cell is "induced" to make virus as a consequence of some provocation. The site of latency may again be local or distant from the site of clinical recrudescence, and the viral genome may either be integrated into the cellular DNA, or virus replication may be blocked in some way without integration into the host cell genome.
Needless to say, there are proponents of both hypotheses, and it will be well to consider them in turn.

The major problem with the dynamic state hypothesis is that Herpes simplex virus is normally cytolytic. As neurons do not multiply, an infection would soon exhaust all available cells. Conceivably, such an infection could be maintained in the deeper layers of the skin epithelium, where the cells do constantly multiply to maintain the epidermal structure (Burnet, 1955). An alternative solution could be that the pathogenesis of the virus may be altered in some way, and it may no longer initiate the events which would normally lead to cell destruction. Even in such a case, however, if viral antigens were being expressed on the cell surfaces, one would expect this to provoke destruction of the cell either by killer cells or by the concerted action of antibodies and complement, for, as pointed out by Roizman (1974), the patients are not immunodeficient.

A strong piece of theoretical evidence in favor of the dynamic state hypothesis is the continuing high level of antibodies found in many subjects. Continuous production of low levels of virus would provide the antigenetic stimulus to maintain these antibody levels (Burnet and Williams, 1939).

Favoring the dynamic hypothesis, Baringer and Swoveland (1973, 1974), reported that if they used exhaustive search methods, they were able with the electron microscope, to find scattered ganglion cells containing Herpes simplex virus in the trigeminal ganglia of rabbits infected via the cornea some months previously. Unfortunately for the
dynamic state hypothesis, there is no proof that they did not by chance just come upon a recrudescence of virus previously in the static state.

Similarly, Schwartz et al. (1978) were able to find intact virus in cell-free homogenates of sacral dorsal root ganglia of mice for up to 8 months after initial infection when they tested the homogenates on organotypic cultures of fetal mouse dorsal root ganglia, but they were not able to find intact virus when they tested the homogenates on HeLa cells. The implications of this work are that virus is being produced continuously, and that the normal failure to find it without organ culture or co-culture of intact cells is not due to the virus being in a noninfectious state, but simply due to the insensitivity of the test systems used. While this is an important concept and deserves further investigation, it has to be said that free virus might again be a recent recrudescence of virus previously in the static state.

Further support for the dynamic theory comes from the work of Yamamoto et al., 1977. While the authors were not able to find infectious virus in cell-free homogenates of the dorsal root ganglia of mice infected with Herpes simplex virus for more than 14 days previously, they were able to detect thymidine kinase for up to 60 days, implying that at least part of the viral genome was being continually or intermittently expressed. Again, however, there is always the possibility that the authors were witnessing a recrudescence from the static state, or that, as Puga et al. (1978) pointed out, the thymidine kinase had a long half life and was simply residual.

Against the idea of a dynamic infection of the neurons, with shedding
of virus in the trigeminal or other ganglia, it may be asked why the infection doesn't spread through the ganglia to infect all the cells, with subsequent recrudescence to areas of skin not previously infected. Anyone who suffers from labial cold sores knows that this does not happen.

For the static state hypothesis, it is an advantage that Herpes simplex virus is a DNA virus. It could, therefore, be integrated into the host cell genomes without any need for a reverse transcriptase. Another point worth mentioning is that neurons do not divide and so may be able to function without some enzymes which would be required both by dividing cells and for replication of virus. Of all the cells in the body, these may, therefore, be most suited for residence in the static state of a virus, being perhaps chemically the ones most suited and in addition, the ones most likely to survive for the life of the host organism. If, however, the lack of some enzyme is in fact necessary for the establishment of a latent state, the problem then of course arises, as to how the virus is eventually able to escape from such cells.

There is much work which supports the static state hypothesis. The numerous failures to find free virus in human trigeminal ganglia (Burnet and Lush, 1939; Richter, 1944; Carton and Kilbourne, 1952; Bastian et al., 1972; Baringer and Swoveland, 1973, 1974; Rodda et al., 1973; Plummer, 1973), and in the ganglia of various animal species (Stevens and Cook, 1971, 1974; Benda et al., 1973; Knotts et al., 1973; Baringer and Swoveland, 1974; Price et al., 1975; Martin et al., 1977) except after organ culture all indicate that there is no continuing active infection. The nagging doubt must, however, remain that perhaps a small amount of virus
which was present in the organ was destroyed during extraction and not
detected subsequently by the detection system.

In addition to these searches for virus, attempts have been made to
find subviral particles during latency. Puga et al. (1978) used the
hybridization technique with radioactive probes to look for viral DNA
and mRNA in the trigeminal ganglia of mice. They found both during the
acute phase of infection, but only DNA in the latent phase. As the con­
tinuous production of virus required in the dynamic state hypothesis
would require the continuous production of mRNA, they argued that this
was evidence that the virus was in a nonreplicating static state. They
did, however, point out that technical limitations prevented their de­
tection of mRNA at a level of less than 400 molecules per ganglia. It
is, therefore, conceivable that low level synthesis could have been
taking place undetected.

Hill and Blyth (1976) pointed out two difficulties with the static
state hypothesis. The first was that viral production is intermittent
and so antigenic stimulation of the immune system is rare. In such cir­
cumstances, one would expect declining antibody levels to occur, with
boosting at times of recurrent infection. In practice, the titer of
antibodies usually remains constant for long periods and is unaffected
by recurrent lesions (Douglas and Couch, 1970), which suggests frequent
antigenic stimuli irrespective of the frequency of the clinical lesions.
The other difficulty is that if it is accepted that the static latent
state of the virus is in neurons, it is difficult to understand how a
superficial stimulus such as ultraviolet light might provoke a
recrudescence. To account for these difficulties, they proposed the "Skin Trigger Hypothesis", which is a variant of the dynamic state hypothesis. It proposes that intact virus is often produced in the trigeminal ganglion and reaches the skin via the nerves almost continuously. In the skin, microfoci of infected epidermal cells develop, but they are usually eliminated by host defense mechanisms, and so most of these infections are abortive. Occasionally, however, changes occur in the skin (hence "skin trigger hypothesis") which allow the microfoci to develop into visible lesions. This hypothesis explains how a cutaneous stimulus could provoke a cutaneous reactivation of a virus latent in the trigeminal ganglion, and also explains how antibody levels could be maintained for long periods. It does not, however, explain the difficulty in finding virus in the skin.

A further aspect of this phenomenon of latency which should be considered is that all cells in a tissue are not necessarily the same, and their interreactions with invading virus may differ. Walz et al. (1976) speculated that a ganglion may contain two populations of cells both susceptible to Herpes simplex virus, but in different ways, with one being lysed and the other being capable of maintaining a latent infection. Sekizawa et al. (1980) made a similar suggestion, in that ganglia may contain two populations of cells, one permissive and the other non-permissive. The host's immune response would detect the viral antigen in the productively infected cells during the acute phase of the infection and eliminate them, leaving only the nonpermissive latently infected cells. Hill and Field (1973) tested cultures of chick embryo dorsal root
ganglia and found that virus replicated in the neurons but not in the glial cells, and that these latter cells showed in them signs of defective viral replication. These authors wondered if the glial cells were the ones which, in fact, harbored the virus in a latent state, rather than the neurons. The dependence of the virus upon a specific and identified cell factor was shown by the observation of Carritt and Goldfarb (1976) that Herpes simplex virus would not replicate in hybrid Chinese hamster x human cells unless chromosome 3 was present.

The significance of these observations is not yet clear. There is, however, obviously considerable scope for further work in this area.

As yet, it has not been stated with confidence whether latent Herpes simplex virus is resident in the human being in nervous tissue or in epidermal tissue, nor is it known whether it exists in a persistent always-multiplying form or in a static quiescent form; thus, it may appear to be presumptuous to even consider discussing the mechanism of control of recrudescence.

It is well-recognized that acute infection with Herpes simplex virus is controlled by both the humoral and the cellular immunological defense mechanisms, and that it is these defenses which prevent run-away infection on first exposure to the virus, or on subsequent recrudescence (Montgomerie et al., 1969; Merigan, 1974; Price and Schmitz, 1978). It would, therefore, appear logical to assume that recrudescences of Herpes simplex virus would occur when immunity waned, and that each recrudescence would induce an immunological response which would protect that individual for a period of time until the immunity again waned.
Unfortunately for this simple hypothesis, it is well-established that there is little correlation between the level of circulating antibodies and the incidence of recurrent disease in the human being (Scott, 1957; Cesario et al., 1969; Douglas and Couch, 1970), and that the titer is not affected by recurrent lesions in the human patient in ordinary clinical recrudescences (Douglas and Couch, 1970).

The situation is similar with the recrudescences which may be associated with trigeminal section of the human being. Ellison et al. (1959) noted that no significant difference was observed in the titer of neutralizing antibody or of complement fixing antibody between patients who developed herpes lesions and those who did not, that the antibody response to the recrudescences was irregular, and that there was no apparent correlation between the severity of the recrudescences and the pre-operative antibody levels.

Blank and Haines (1973) noted that it was easy to reinfect human patients on the skin, and so speculated that the epithelia of the skin, eye and cervix were immunologically privileged.

The amount of experimental work performed with laboratory animals to investigate immunological control of latency has been quite limited, and its significance is not yet clear. Stevens and Cook (1974) transplanted latently infected spinal ganglia from mice into syngenic animals, and found that the administration of IgG prevented viral reactivation. Price et al. (1975) found that it was possible to establish latent infections in mice despite the presence of high titers of neutralizing antibody, but Walz et al. (1976) using the same animal species noted that
antibodies affected the number of ganglion cells infected at primary infection, and that this in its turn affected the number of latently infected cells. Burnet and Williams (1939) believed that the continuing high levels of antibodies in human beings were caused by the frequent recrudescence of virus even in the absence of clinical signs. Hill and Blyth (1976) took this further and speculated that they were due to the constant release of small amounts of virus from nerve endings to form microfoci of infection in the skin, and they proposed their Skin Trigger Hypothesis to account for the fact that only occasionally did these develop into clinically-visible lesions.

An interesting observation made by several observers and reviewed by Westmoreland and Watkins (1974), was that cells infected with Herpes simplex virus develop Fc receptors on their surfaces. This observation was noted by Lehner et al. (1975). These investigators believed that immunoglobulins play a vital part in both the protection of neurons infected with virus and in the repression of viral multiplication in the cells. They postulated that in infected neurons, viral antigens are expressed on the surface, and that specific immunoglobulins bind to this by their Fab portions, and that the same molecules of immunoglobin also bind to Fc receptors on the same cells by their Fc portions, thereby forming bridges. This bridging serves to mask the viral antigens, and so protects the cells from destruction by killer cells or antibodies acting with complement. In addition, the authors postulated that this bridging in some way which is as yet not understood, suppresses the virus and prevents its multiplication and its ability to destroy the infected
cells. Recrudescence would occur when the immunoglobulin bridges were lost.

Sekizawa et al. (1980) were, however, not convinced of the importance of immunoglobulins. They infected mice and then immediately passively immunized them so as to suppress their own active immunological responses. At a later date they were able to recover latent virus when they were no longer able to detect circulating antibodies. From this, they argued that latency can exist in the absence of antibodies. Against this it may be suggested that being unable to detect circulating antibodies does not necessarily mean that there are no antibodies present, and that if the hypothesis of Lehner et al. (1975) is correct, there need be no circulating antibodies to maintain latency. All that is required is antibody bound to the infected cells.

If humoral antibodies do control the state of latency, then immunosuppression might be expected to produce recrudescence. Indeed, this has been reported by a number of workers. Montgomerie et al. (1969) noted recrudescence of active Herpes simplex virus infection in a number of patients after renal transplants. It is possible that this was precipitated by the immunosuppressive drugs used in such procedures, or it might have been precipitated by other factors, such as surgical shock, acting by mechanisms which are not yet understood. Russell (1974) used adrenocorticotropic hormone together with antilymphocyte therapy to treat multiple sclerosis. He noted recrudescence of active Herpes simplex virus infections, some quite severe, in 6 of 10 of his patients. Again, these could have been caused by the depression of the immunological
system, or, alternatively, as Russell pointed out, they could have been precipitated by the concomitant fever associated with the treatment.

Other workers, Underwood and Weed (1974); Robinson and Hurd (1975); Price and Schmitz (1978); Openshaw et al. (1979); Sekizawa et al. (1980), tested the effect of cyclophosphamide and hydrocortisone in mice and were able to provoke recrudescence of active *Herpes simplex* virus infections. Unfortunately, the mechanism of this effect is totally unknown and interpretation is therefore difficult. Such treatments make the test animals ill and permit the development of intercurrent disease. As *Herpes simplex* recrudescence can be provoked by a wide range of stimuli, it is quite possible that the experimental chemical agents did not act by direct immunological mechanisms, but by making the animals ill and thereby acting by indirect mechanisms which are not yet elucidated.

In contrast to the successful recrudescences of active *Herpes simplex* virus infections described above, Blyth et al. (1976) were not able to induce clinical signs of recrudescence in the mouse ear by the use of immunosuppressive drugs. This, however, does not prove that the drugs had no effect at all. It may merely mean that the test system was not particularly sensitive. It must, however, be said that these authors were able to get recrudescence by using prostaglandin E₂ (Hill and Blyth, 1976; Blyth et al., 1976), leading the authors to speculate that this substance may be an important factor in their skin trigger hypothesis.

In concluding this section, it has to be said that despite the substantial amount of work which has been done in this area, there is still little knowledge of the manner in which the herpes viruses become latent,
or of the mechanisms which control this latency. This subject is of substantial theoretical and practical importance and it is to be hoped that its study will be pursued energetically in the coming years.

The Establishment of Latency in Vitro with Herpes simplex Type 1 and Pseudorabies Viruses

Over the last twenty years many investigators have tried to establish in vitro models of viral latency using many of the families of viruses. Such models would have many advantages over their in vivo prototypes, the chief of which would be the possibility of removing from the system individual components, so that the effect of their loss could be observed, a procedure which is difficult or impossible in vivo. The large volume of experimental data which has accrued from this work has been reviewed by Huang and Baltimore (1970), by Stevens et al. (1978), and by Fraenkel-Conrat and Wagner (1980).

Many investigators have been able to establish persistent infections in in vitro cultures of cells, but whether this persistence simulates latency in whole animals or whether it is an unrelated phenomenon, is not yet clear. In this text, it will be referred to as persistence, rather than latency, because of this doubt.

Various mechanisms of persistence have been proposed for in vitro systems. These include:

1) Defective interfering particles (Huang and Baltimore, 1970)
2) Temperature sensitive particles (Preble and Youngner, 1975)
3) Integration of the genetic information of RNA viruses into the cell genome using reverse transcriptase (Simpson and Iinuma,
The Herpetoviridae have not been neglected by investigators; *Herpes simplex* in particular having been tested in a variety of systems.

Hampar and Copeland (1965) tested the susceptibility to *Herpes simplex* virus of Chinese hamster lung cells from their first passage until in excess of 60 passages. They noted that after the 10th cell passage it took longer for the virus to destroy the cells, but that it was still completely cytocidal up until the 30th passage of the cells. When cells had been serially passaged for more than 30 times, however, they were no longer completely destroyed by infecting virus and persistent infections were established. These persistent infections were cyclical, in that there were periods of almost complete cell destruction, followed by periods of remission, in which cells regrew. Each cycle took about 16 days, and as the investigators were able to maintain the system for 6 cycles, the persistent infections lasted for in excess of 100 days. Further study of this system showed that less than 0.1% of the cells remained at the end of a cycle of cell destruction (Hampar, 1966), and that with time there were changes in both the cells and the virus. The cells increased in resistance to the virus, and the virus in the system increased in virulence for these resistant cells (Hampar and Burroughs, 1969).

Nii (1969, 1970), using Earle's L Cells, was able to establish a persistent cycling infection with *Herpes simplex* virus continuing for four years. He noted that in his system there were produced many incomplete virus particles which lacked envelopes and cores.
Szántó et al. (1976) noted that persistent infection could be established in vitro in some cell types but not in others. They were able to establish persistent infections in BHK-21 cells for in excess of 297 days and 36 cell passages, but could not establish persistent infections in HeLa cells or in ZP rabbit lung cells. The persistently infected BHK-21 cell cultures were resistant to superinfection.

An interesting change was noted by Doller et al. (1979) in the persistent infections which they established in cells derived from a rat central nervous system tumor. They were able to recover infectious virus from this system for up to 57 cell passages, but thereafter they were only able to recover herpes-specific proteins and noninfectious structures which looked like herpes virions when examined by using the electron microscope. The authors speculated that the cells were now either carrying an incomplete *Herpes simplex* genome, or else were carrying the full genome but only expressing part of it.

The importance of the state of the cells was shown by Dunn et al. (1979) when they established a cyclical persistent infection in BHK-21 and Balb/3T3 cells for up to 9 months. They noted that the cells had to be in the S-phase (that is synthesizing DNA) to survive infection.

In addition to these experiments which involved only virus and cells, a number of investigators have tested the effects of antibodies on the system. Hoggan and Roizman (1959) were able to maintain persistent *Herpes simplex* infections in FL cells for 20 passages in the presence of antibodies. Wheeler and Canby (1959) were not able to establish persistent infection in HeLa cells alone, as was confirmed by Szántó et
The viral infections spread relentlessly and destroyed all the cells. When, however, they added specific neutralizing antibody to the system, the cultures were not destroyed and could be passaged serially, apparently indefinitely, and the virus infection persisted. Szántó (1963) was also successful in establishing persistent infection in HeLa cells (and in Detroit 6 cells) when he added antibodies to the system. Fernandez (1960) noted that HeLa cells infected in the presence of antibodies increased in resistance (X10 - X100) compared with the original cells when tested with virus from the persistent system, and Hinze and Walker (1961) noted that the virus altered in its characteristics after passage in HeLa cells, human conjunctiva cells and KB cells in the presence of antibodies. Its cytopathogenicity changed and it lost its virulence for mice.

Perhaps the closest in vitro approximation to in vivo latency was reported by Nii (1970). He noted that after establishing infection in Earle's L cells in the presence of antibodies, that when the cells were subcultured in the absence of antibodies, cytopathic effect and the production of infectious virus were only detected in some cases after a lag period of several months. He also noted that it was very difficult to eradicate in vitro infections using antibodies.

The role of interferon has been considered by a number of investigators. Glasgow and Habel (1963) noted that Herpes simplex virus alone would simply destroy a mouse embryo cell line, but in the presence of polyoma virus a persistent infection of the cells was established with both viruses at the same time, that is, a double carrier culture. They
speculated that the survival of the culture in the presence of Herpes simplex virus depended upon the production of interferon by cells infected with the polyoma virus, but they were not able to establish persistent Herpes simplex virus infections in polyoma virus-free cells by adding interferon. Waddell and Sigel (1966) believed that the critical factor in the long-term protection of KB cells from Herpes simplex infection was a substance with several of the characteristics of interferon. This substance was found to be produced when the entire experiment was conducted at 35°C, but not when the experiment was started at 25°C. In the latter case, when the temperature was raised to 35°C, the cells were destroyed, even if antibodies were added to the system. Szántó et al. (1976), however, when they established persistent infection of BHK cells in the absence of antibodies, were of the view that interferon was not required in the system, unless it was acting at undetectably low levels.

The effects of more drastic chemical manipulations than either antibodies or interferon upon the cell-virus relationships have been tested. Cytosine arabinoside (ara-C) inhibits DNA synthesis by interfering with the reduction of cytidine diphosphate. O'Neill et al. (1972) tested its effect upon a culture of human embryonic lung cells infected with Herpes simplex type 2, and noted that it stopped the cytopathic effect and the production of the virus. When the ara-C was removed from the culture, there was a delay of 4-5 days before virus production was resumed. Whether this not unexpected delay was in any way related to in vivo latency, or to the in vitro persistence reported by other investigators, is a matter for debate. To support their contention that this
4-5 day lag was a type of persistent infection, the authors pointed out that during this period the cultures were sensitive to superinfection, so the cells had presumably recovered from the effects of the ara-C. A difficulty with this argument, however, is that cells which have suffered two insults, namely invasion by virus and disturbance of function by ara-C, may take longer to recover than do cells which have only been affected by the ara-C. The former cells may, therefore, take longer to produce virus again than do cells which have their first encounter with virus during the challenge.

Several investigators have tested the effects of manipulation of temperature upon the cell-virus system. Kelleher et al. (1975) maintained primary rabbit kidney cells infected with *Herpes simplex* virus type 2 at 41°C for five days and noted that infectious virus could not be recovered from them even after disruption by freezing and thawing or by sonication. When the temperature of similarly cultured still intact cells was lowered to 37°C, spontaneous reactivation of infection occurred in 84% of the cultures, with lag periods of up to 45 days. Coleman and Jawetz (1961) tested the effects of *Herpes simplex* type 1 upon cells in culture derived from a lung carcinoma and maintained at 31°C. They noted that persistent infection developed lasting for up to 18 months, and that it was accompanied by continuing high titer of virus in the supernatant fluids, indicating that the low temperature was not simply disrupting viral multiplication. When the temperature of the culture was raised to 37°C, the cells were destroyed by the virus.

Whether these temperature-related phenomena are in any way related
to in vivo latency is not known. A temperature of 41°C may occur in a mammal for a short time without irreparable damage. It is conceivable that such a temperature, either caused by the viral infection itself or coincidental from another cause, might induce latency, but there is to date no evidence at all to support this suggestion. The lower temperature, 31°C, is not likely to occur in any mammal except during hibernation and so needs hardly to be included in this discussion.

Compared with Herpes simplex virus, the amount of work which has been done with pseudorabies virus is very limited. Golais et al. (1978) obtained results similar to those obtained by Kelleher et al. (1975), discussed above, when they infected BHK-21 cells adapted to 40°C culture with pseudorabies virus. Cytopathic effect did not occur in the cultures and virus could not be recovered from cells disrupted by freezing and thawing. If, however, after 4 or 5 days similarly prepared but intact cells were trypsinized, divided and repassaged at 37°C and 40°C, respectively, virus could be recovered after 2 or 3 days from the cultures at 37°C, but not from those at 40°C. It will be noted that in this report the lag period was much shorter (2 or 3 days) than that recorded by Kelleher et al. (1975) using Herpes simplex type 2 virus (up to 45 days).

Golais and Sabó (1979) also tested the effect of ara-C on the growth of pseudorabies virus in Vero cells, and noted that it completely inhibited the multiplication of the virus. When the ara-C was removed from the system, virus growth was resumed after 3-5 days. As with the work which was done with Herpes simplex virus, it can only be commented that the relationship, if any, of these temperature and ara-C induced phenomena
to latency in vivo or persistence in vitro is not known.

In conclusion to this discussion of attempts to establish latent herpetic infection in vitro, it can only be said that with the possible exception of the reports of Nii (1970) and Kelleher et al. (1975), the models described do not convincingly simulate in vivo latency. Nevertheless, the potential value of a good model justifies further effort in this area.

The Latency of Aujeszky's Disease Virus

Aujeszky's Disease (pseudorabies) virus is a member of the Herpetoviridae. The virus appears to be best adapted to the pig and may, particularly in older animals, not cause any noticeable clinical signs. In young pigs, however, it may cause a severe rhinitis, pneumonia and encephalitis, with high mortality. In recent years, strains of higher virulence than hitherto recognized have been found to cause severe clinical illness in older animals in various parts of the world. The virus may also affect other mammalian species; the disease which it causes in them if often characterized by severe puritis which may lead to self-mutilation, hence the layman's name for the disease, "Mad Itch". The host range and clinical signs of this disease have been reviewed by Davies (1979).

Other members of the Herpetoviridae such as Herpes simplex and Varicella/Zoster are capable of entering into quiescent latent states in which they exhibit no clinical signs in their hosts, often for years, and then recrudesce to give recognizable clinical disease with shedding of
infectious virus (Fenner et al., 1974). As pseudorabies virus appears to be a typical member of the Herpetoviridae, it is to be expected that it may do the same.

The first evidence of recrudescence of pseudorabies virus from the latent state was epidemiological. In Russia, Nikitin (1961) noted that pigs, especially sows which had had the disease and which appeared to be fully recovered, could be instrumental in introducing the disease onto other farms months later. In the United States, Howarth (1969) was convinced that apparently healthy pigs could shed the virus and infect other pigs, and that climatic stress was an important factor in the initiation of this recrudescence. This work was supported by Simeonov (1973) who provided evidence that pigs clinically convalescent from pseudorabies for at least 60 days could infect healthy pigs placed in contact with them, and by Beran et al. (1976) who believed that the episodes which they investigated provided some evidence for carrier states of up to 4 months in pigs. These authors also suggested that stress of movement, farrowing or lactation appeared to play a role in transmission.

The problem with epidemiological evidences of recrudescence in field situations is that one usually cannot be absolutely certain that infections were not introduced from some other sources. Clearly it would much strengthen the case for recrudescence if virus could be found in carrier animals. This was first reported by Nikitin (1961), who was able to demonstrate virus in various tissues of pigs for up to 6 months after their recovery from clinical disease, and by Simeonov (1973), who was able to recover virus from the tonsils of pigs for up to 4 months after
their recovery from clinical disease. Following the success of Stevens and Cook (1971) in using organ cultures to recover *Herpes simplex* from mouse tissue, it was natural that this technique should be used by workers with pseudorabies. Sabó and Rajčáni (1976) were able to recover virus in the supernatant fluids of explants of tonsils, cervical lymph nodes, nasal mucosa and trigeminal ganglia for up to 6 months after infection, and Beran et al. (1980) were able to recover virus in the supernatant fluid of trigeminal ganglia for up to 13 months after infection. In addition to this recovery of intact infectious virus, Gutekunst (1979) was able to detect viral DNA in pig tissue for up to 7 months after clinical infection by using RNA/DNA hybridization techniques.

The work to this point had, therefore, shown quite clearly that pseudorabies virus could be retained in the pig for considerable periods of time, and it had suggested that the virus could be shed and infect other animals. It was natural that workers should seek to show this latter phenomenon.

McFerran and Dow (1964) attempted to do this by stressing pigs. They sensitized them with egg albumen, infected them with pseudorabies, and then 48 days later, when they were fully recovered, they provoked anaphylactic reactions in the animals by the intravenous injection of more egg albumen. While the anaphylaxis which was produced by this procedure was in some cases sufficiently severe to stop respiration and require the animals to be resuscitated, it did not induce the pigs to shed pseudorabies virus in their nasal or oral secretions. At a later date, some of the pigs were fed on a diet containing a very high level of soya
meal. This caused a severe diarrhea from which a pure culture of hemolytic *Escherichia coli* was isolated, but again the pigs did not shed pseudorabies virus in their nasal or oral secretions.

Sabó (1969) also tried to induce recrudescence of pseudorabies virus by stressing pigs which had been infected 31 days previously and which had fully recovered. He injected them intravenously with cortisone, and then in the subsequent 36 days subjected them to stress by spraying them on 3 occasions with water at 12°C for 10 minutes, by keeping them in a cramped poorly ventilated space at 24°C for 3 days, and by feeding them only once a day with a small ration of liquid food. At no time was he able to recover virus in swabs of the throat.

Crandell et al. (1979) were, however, more successful. They vaccinated pigs with an attenuated pseudorabies vaccine and then challenged them with fully virulent virus. Approximately 90 days later they injected the pigs with dexamethasone by the intramuscular route daily for 4-6 days. By this technique, they were able to recover pseudorabies virus from the nasal secretions of 5 of the 6 pigs tested.

The experiments described in this dissertation were designed to continue these lines of enquiry by investigating whether there was any association between farrowing and recrudescence (Nikitin, 1961; Beran et al., 1976), by considering the cause of the repeated seroconversions which had occurred for several years on a farm in northern Iowa, and by attempting to establish persistent infection of pseudorabies virus in porcine tissue fragments maintained in vitro. The first of these aims was successful in that virus was recovered from the nostrils of a sow in
the postparturient period 19 months after she had been clinically infected. These data have been published (Davies and Beran, 1980), and are recorded here in the first paper. This success naturally led to attempts to repeat the procedure, as described in the second paper, and to an investigation of the effect of hormones in pregnancy and parturition, reported in the third paper. In the herd investigated in northern Iowa, pseudorabies virus was not recovered from the sows in the breeding unit (Davies, unpublished data), but was recovered from a pig so young as to suggest transplacental transmission. The characterization of this virus showed it to be of low virulence and quite unlike previous isolates in this geographical area; therefore, of epidemiological interest as discussed in the fourth paper. The final paper describes the successful establishment of persistent infections by pseudorabies virus in vitro.
SECTION I. SPONTANEOUS SHEDDING OF PSEUDORABIES VIRUS FROM A CLINICALLY RECOVERED POST-PARTURIENT SOW
Spontaneous shedding of Pseudorabies virus from a clinically recovered post-parturient sow

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SUMMARY

Pseudorabies virus was shed by a sow that had been clinically infected 19 months earlier (when 1 month old) and had been in isolation for 3½ months. The shedding was detected from the 3rd to 8th days after farrowing and was confined to the nasal secretions. Two susceptible pigs in contact with the sow after farrowing were not infected.
INTRODUCTION

Pseudorabies virus has been shown to persist in the tissues of previously infected and clinically fully recovered pigs for periods of up to at least 13 months (Nikitin, 1961; Sabó and Rajčáni, 1976; Beran et al., 1980). It has been suspected for some time that the virus may be shed on occasion, particularly by swine in breeding units (Nikitin, 1961) subjected to climatic stress (Howarth, 1969), or subjected to the stress of movement, farrowing, or lactation (Beran et al., 1976).

Such suspicions have been based upon otherwise unexplained occurrences of pseudorabies in circumstances when it was not possible to identify any other potential source of infection. While such suspicions may be cumulatively convincing, individually they are suspect, for there is no proof that virus was not introduced into a herd by a route that was not identified by the observer or, in some cases, that it was not a flaring-up of a previously unnoticed circulating subclinical infection.

The only proof of shedding from a persistently infected pig is the detection of shedding from a pig that has been in isolation for some months.

Various attempts to induce shedding from pseudorabies convalescent pigs through artificially induced anaphylaxis and diet changes (McFerran and Dow, 1964) or by cortisone treatment and temperature and diet changes (Sabó, 1969) have been unsuccessful. Recently, however, Crandell et al. (1979) reported experimental recrudescence of shedding in pigs. They vaccinated weanling pigs, challenged their immunity with virulent virus and subsequently treated them with dexamethazone.

In this report, the shedding of virus occurred without immunosupressant treatment.
MATERIALS AND METHODS

The pig was a Hampshire-cross from a pseudorabies-free herd. The pig, its dam, and its littermates, had no detectable neutralizing antibodies to pseudorabies and had no history of vaccination against the disease. It was infected at the age of 1 month by the instillation into its nostrils of fluid containing the 7th cell culture passage of strain S62/26 ("Iowa" strain, "Wilson" strain) of virus, to give a total dose of $10^7$ plaque forming units, and developed typical symptoms of pseudorabies disease. On recovery, it was left in quarantine, with contact only with similarly treated littermates and with 2 other pigs which were part of another experiment. These 2 pigs had been vaccinated 4 months previously with a proprietary attenuated vaccine and challenged 2 months later with strain S62/26. They were clinically normal when the groups were put together to economize on animal accommodation.

When the pig was 16 months old, it was artificially inseminated with semen derived from a boar maintained in a herd known and tested to be clear of pseudorabies and from which semen had been tested and found to be free of virus. A week later the pig was put into an isolation pen, where it was kept for the duration of the experiment. Three days before farrowing, 2 sentinel pigs of 1 month of age, derived from a pseudorabies-free herd and themselves seronegative were placed in a cage in the pen with the gilt. These pigs had intimate contact with the gilt's feces and

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¹Norden Laboratories, P.O. Box 80809, Lincoln NE 68528.
urine, and when she farrowed, with the placenta and vaginal discharge. Two days after farrowing, the sentinel pigs were released from their cage and allowed to run with and suckle the sow.

Each day for 14 days, swab specimens were obtained from the nasal mucosa of the sow, from several of her own pigs, and from the 2 sentinel pigs, as well as from the sow's rectum and vagina. All specimens, together with samples of her milk, were tested for virus in Madin Darby bovine kidney (MDBK) cells in tubes, using standard methods. When the results were positive, the identity of the virus was confirmed by use of serum neutralization tests.

The farrowing was complicated by the gilt's inexperience in operating the water bowl in the farrowing crate, leading to a measure of dehydration until she was removed from the crate on the day after farrowing. This dehydration was accompanied by moderate pyrexia (40.9 °C) and decreased milk production. Treatment with gentamicin sulfate\(^1\) and oxytocin\(^2\) and access to adequate water supply resulted in rapid recovery by the next day.

On the 22nd day after farrowing, the 2 sentinel pigs, and 2 pigs of the sow's own litter were removed to a separate isolation unit and with them were placed 2 more pigs derived from the same litter as the sentinel pigs. Sera from these 6 pigs and from the sow were tested for neutralizing antibodies. The immunity of the 6 pigs was challenged by swabbing

\(^1\)"Gentocin." Schering Corporation, Kenilworth, NJ 07033.

\(^2\)Nelson Laboratories, Sioux Falls, SD 57101.
and slight abrasion of the nares with a solution containing $10^6$ plaque forming units of the 9th tissue culture passage of S62/26 strain virus. The pigs were examined daily and their clinical conditions were assessed.
RESULTS

After infection with pseudorabies virus, the test gilt had signs of moderately severe disease but made an apparently complete recovery without treatment. At 1 month after infection, her serum neutralizing antibody titer to pseudorabies virus was 1:32.

At the time of farrowing (19 months later), the gilt was clinically normal and all exudates were test negative for virus. Her serum neutralizing antibody titer was 1:4 on the first day after parturition.

On days 3 through 8 after farrowing, virus was detected in the sow's nasal secretions. It caused cytopathic effect typical of pseudorabies in MDBK cells and it was neutralized by specific antiserum. Other than the distress and pyrexia that was attributed to inadequate water intake, at no time were there any signs of illness. Virus was not detected in vaginal secretions, milk, or by rectal swabs. The neonatal pigs in the litter appeared normal and developed normally, except for a transient high-stepping action of a hind limb of one pig. At no time did any pigs of the litter or either of the sentinel pigs manifest signs of pseudorabies, and nasal swab specimens from the litter and the sentinels were consistently negative for virus.

At 22 days after farrowing, the 2 tested pigs from the sow's own litter had serum titers of 1:4 each and the sow herself had a serum titer of 1:64 when tested against 300 tissue culture ID₅₀ of virus. The 2 sentinels did not have detectable antibodies. When their immunity was challenged with virulent virus, the sow's own pigs showed only mild
reaction with pyrexia to 41.4 C and the production of a virus-containing serous nasal discharge for a few days. The sentinel pigs and the 2 freshly introduced pigs, all littermates and now aged 7 weeks, were severely affected by the challenge (pyrexia, purulent virus-containing nasal discharge, and dyspnea). One of the freshly introduced pigs died. The other 3 pigs eventually recovered.
DISCUSSION

The observation that the sow described in this experiment shed virus in the immediately post-parturient period supports the warning of Nikitin (1961), that it is inadvisable to introduce pigs that have recovered from pseudorabies into other herds, especially into farrowing units.

It should be recorded that persistent virus infection had already been noted in 6 of the 7 original littermates of the gilt when tested in another experiment by slaughter and cultivation of tissue explants in nutrient medium (Beran et al., 1980). The finding of persistent infection in the gilt was, therefore, no surprise.

Persistent infection must be distinguished from subclinical infection circulating through a population of animals. The gilt in this experiment had been in isolation for 3½ months prior to farrowing. For this period at least, the infection could not have been in any form other than a persistent infection. For 12 months prior to that period, the gilt had been with 3 other pigs. One of these had been infected with pseudorabies virus and the other 2 had been vaccinated and then challenged with virulent pseudorabies virus as part of another experiment. While it is presumably possible for infection to be maintained in a large herd of pigs by circulation amongst individuals with waning immunity, it is highly unlikely that a subclinical circulating infection could have continued in this small group. For 4 months prior to that period, the gilt had been with a small group of littermates convalescing from an acute infection, when presumably immunity was at its highest. Again, it
seems unlikely that reinfection from a circulating subclinical infection could have occurred in this period. The gilt had, therefore, certainly carried the infection in a persistent form for 3½ months and had probably carried it in a persistent form for 19 months.

Prior to this experiment, it had been anticipated that if the gilt did shed virus, nonimmune contacts would become clinically infected. In this experiment, the 2 sentinel pigs did not develop clinical disease and they did not produce neutralizing antibodies. On challenge of immunity, they were severely affected. The reason for this is not known.

The rise in titer of circulating antibodies in the gilt from a low level at the time of farrowing to a titer of 1:64 after the period of shedding is of some interest. If this is the regular pattern, it may indicate a practical means of distinguishing pigs that have shed virus at parturition, even though they and their litters had no clinical disease, and even if they were not, as in this case, a source of infection to sentinel pigs. Clearly, this phenomenon warrants further study.
REFERENCES


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SECTION II. FURTHER OBSERVATIONS CONCERNING THE SHEDDING OF PSEUDORABIES VIRUS IN A POSTPARTURIENT SOW
Further Observations Concerning the Shedding of Pseudorabies Virus in a Postparturient Sow

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Further studies are described on a sow which had shed pseudorabies virus in the postparturient period of her first farrowing. The animal was not observed to shed virus after the two subsequent farrowings. The changes in her neutralizing antibody titer associated with farrowing are described and discussed, and the relationship of the hormonal and chemical events which take place at parturition and recrudescence of virus shedding are briefly reviewed.
INTRODUCTION

Most, if not all, members of the Herpetoviridae are able to cause persistent or latent infections in their hosts for long periods of time without causing clinical signs. On occasion the infection may recrudesce by mechanisms not yet understood and clinical disease may result. Herpes simplex type 1, for example, may recrudesce repeatedly at the same site in an individual.

Pseudorabies (PR, Aujeszky's Disease) virus appears to be a typical member of this group in that it has been found in the tissues of clinically recovered pigs for periods of up to 13 months after initial infection (Nikitin, 1961; Sabó and Rajčáni, 1976; Beran et al., 1980) and part at least, of its genome has been found for seven months (Gutekunst, 1979). Shedding of pseudorabies virus has been artificially induced by the administration of dexamethasone (Crandell et al., 1979) and shedding under natural circumstances has been described in the postparturient period of a sow at her first farrowing (Davies and Beran, 1980).

In this report, we describe the outcome of the two subsequent farrowings of this same sow.
MATERIALS AND METHODS

The pig was a Hampshire-cross, one of an experimental group which was infected at the age of 1 month by intranasal instillation of fluid containing the seventh cell culture passage of pseudorabies virus of strain 62/26 ("Iowa" strain, "Wilson" strain). The pig developed typical signs of pseudorabies. On recovery, it was kept in quarantine until it reached maturity, at which time it was inseminated with semen tested to be free of pseudorabies virus. It was then put into single isolation. The subsequent farrowing at 20 months of age was complicated by the sow's inexperience in operating the water bowl of the farrowing crate. This led to a measure of dehydration until the animal was removed from the crate on the day after farrowing. Its temperature reached 40.9°C and its milk production was subnormal. Treatment with gentamycin sulfate¹ and oxytocin² and access to adequate water resulted in rapid recovery by the next day. On days 3 through 8 after farrowing, pseudorabies virus was detected in the sow's nasal secretions. The early history of this animal and its first farrowing have been described elsewhere in greater detail (Davies and Beran, 1980).

Throughout the second and third pregnancies the sow was maintained in single isolation, except for contact with pigs in her own litter and 2 pairs of sentinel susceptible pigs described later. For both pregnancies, she was inseminated with semen tested to be free of pseudorabies virus

¹Gentocin, Schering Corporation, Kenilworth, NJ.
²Nelson Laboratories, Sioux Falls, SD.
and derived from a boar maintained in a herd known and tested to be free of pseudorabies infections. She was permitted to farrow running free in a pen, rather than being confined in a farrowing crate, and had ready access to water at all times. Her second and third farrowings were at ages 26 and 32 months, respectively.

The sow's nostrils were regularly swabbed during pregnancy at approximately weekly intervals and the nasal secretions were tested for the presence of pseudorabies virus by inoculating on tube cultures of Madin-Darby bovine kidney (MDBK) cells. Cultures were incubated at 37°C for four days and observed daily for cytopathic effects typical of PR virus.

After each farrowing, two 4-7-day-old seronegative sentinel pigs derived from a herd known and tested to be free of pseudorabies and themselves seronegative were placed with the sow and allowed to suckle along with her litter. Sera were collected from the sow, pigs in her litters and the sentinels at various times and tested for the presence of pseudorabies neutralizing antibodies against 300 tissue culture ID50 of virus. Nasal secretions, saliva and vaginal secretions were collected from the sow, and nasal secretions were collected from the litters and sentinels each day for 2 weeks after farrowing, and tested for virus in MDBK cell cultures. In an attempt to free virus from possible virus-antibody complexes, nasal secretions collected during the postparturient period of the second farrowing were dialyzed overnight in a membrane dialysis tube1 (6,000-8,000 M.W. cutoff) against a salt-free solution at 4°C before inoculation on to MDBK cells. Nasal secretions were also examined under the electron microscope after negative staining (Ritchie and Fernelius, 1969).

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1 Spectrapor, Spectrum Medical Industries Inc., Los Angeles, CA.
RESULTS

The second and third farrowings were uneventful except in that the sow, not being confined in a farrowing crate, overlaid and killed several of her pigs. She did not become dehydrated or suffer from pyrexia, and did not require treatment with antibiotics or oxytocin.

Virus was not detected in the sow's nasal secretions collected at approximately weekly intervals during the pregnancies, nor was it detected in the sow's nasal or vaginal secretions or saliva collected in the postparturient periods. Similarly, virus was not detected in the nasal secretions of pigs in the litters or of the sentinel pigs placed with them.

Virus could not be detected in the nasal secretions of the sow in the second postparturient period after dialysis, nor could virions or virion-antibody complexes be seen in these same secretions when observed by electron microscopy.

The second and third farrowings had little effect upon the titer of the neutralizing antibodies to pseudorabies in the sow's serum. The titers rose from 1:8 to 1:16 and from 1:4 to 1:16, respectively, during the 22 days following parturition.
DISCUSSION

The sow used in these experiments shed virus in nasal secretions from the third through the eighth day following the first farrowing, as previously described (Davies and Beran, 1980). In the postparturient period of the 2 subsequent farrowings described in this report, no virus was detected in nasal secretions, vaginal secretions or saliva, nor in the nasal secretions of pigs in her litter.

The possibility was considered that virus might have been shed but that it might have been rendered noninfectious by simultaneously excreted antibodies, and that it was, therefore, not detectable in the MDBK cell system used. An attempt was made by dialysis against a salt-free solution to disassociate any virion-antibody complexes which might have existed. This procedure was not successful. In addition, a search was made of the sow's nasal secretion using electron microscopy for virions of the herpes type, or for virion antibody complexes. This again was not successful. Thus, while virus certainly was shed in the sow's nasal secretions after the first farrowing, it is fairly certain that none was shed after the 2 subsequent farrowings. The reason for this is not known.

It is well-recognized that active infection by another of the Herpetoviridae, Herpes simplex virus type 1 (HSV1) of human beings, may recrudesce repeatedly and produce lesions and infectious virus at the same site in an individual. The familiar "cold sore" on the lips is the classic example. It is believed that a wide variety of stresses such as
a common cold, fever, exposure to excessive ultraviolet light and menstruation may play a part in at least some of these episodes (Schmidt and Rasmussen, 1960). In accordance with this theory, it may be suggested that there is normally more stress associated with the first farrowing of a gilt than with subsequent farrowings, and in this particular sow stress was compounded by the problem with the water supply. These factors, perhaps together with the associated medication, precipitated the recrudescence of virus in the postparturient period of the first farrowing, while in the subsequent farrowings there was little stress and the virus, therefore, did not recrudesce. While this theory fits the data, the proof is insufficient.

The sequence of events leading to parturition in pigs is believed to start with the pituitary stimulating the adrenal glands to produce cortisol, which causes release of prostaglandin $P_2 \alpha$, probably from the uterus. The prostaglandin causes luteolysis, release of relaxin and oxytocin from the corpora lutea and the pituitary, respectively, and stimulation of uterine contractions (First and Bosc, 1979). The relationship of these 3 chemicals to recrudescence of herpes viruses is worthy of consideration.

It has been shown that dexamethasone, a synthetic analogue of cortisol, may induce recrudescence of pseudorabies in the pig (Crandell et al., 1979), and of infectious bovine rhinotracheitis, another of the Herpetoviridae, in cattle (Sheffy and Davies, 1972). It has also been shown that prostaglandin $E_2$ may induce recrudescence of Herpes simplex type 1 when injected into the ears of mice previously infected with the
virus at that site (Blyth et al., 1976). This latter observation led Hill and Blyth (1976) to propose a "skin trigger theory" of recrudescence, in which they postulated a continuing or frequent release into the skin of virus from the ganglia of the central nervous system. They further postulated that this virus is normally destroyed by the host, but sometimes under the influence of prostaglandins, it causes clinical disease. Prostaglandins are found at various times in many tissues, including damaged tissues and menstrual fluid. The relationship of recurrent oral infections of *Herpes simplex* type 1 and menstruation has been noted (Schmidt and Rasmussen, 1960). Oxytoxin is produced at parturition and stimulates uterine contractions and milk let-down. To the best knowledge of the authors of this paper, it has not been deliberately tested as a recrudescing agent in herpetic infection. In the present report, however, oxytocin was used for therapeutic purposes during the first farrowing of the sow when there was shedding of virus, and it was not used in the 2 subsequent farrowings, when virus was not shed. While this is worth noting, it may simply be a coincidental observation.

In summary, two of the chemicals involved in parturition (cortisol and prostaglandin), have been shown experimentally to be capable of inducing recrudescence of herpes viruses, and the third (oxytoxin) was used in therapeutic doses in the only reported case of recrudescence of pseudorabies virus in a sow at parturition. Clearly there is need for further investigation of the relationship of these chemicals to herpetic recrudescence.

It is believed that the frequency of recrudescence of active
infection by Herpes simplex in human beings is not influenced by the level of circulating serum neutralizing antibodies, whether it be high or low (Douglas and Couch, 1970). The sow's serum neutralizing antibody titer to pseudorabies was 1:4 at first farrowing, when she shed virus, and was essentially the same, 1:8 and 1:4, on the 2 subsequent farrowings, when she did not shed virus. It would seem, therefore, that the recrudescence of active pseudorabies infection in the sow at parturition may follow the same pattern as Herpes simplex in human patients and may not be predicted by observing serum neutralizing-antibody levels at the time of farrowing.

Recurrent Herpes simplex disease in human patients is not usually associated with changes in serum neutralizing antibody levels (Scott, 1957; Cesario et al., 1969; Douglas and Couch, 1970). When pseudorabies virus shedding occurred at the sow's first farrowing, her serum neutralizing antibody titer rose from 1:4 to 1:64 by the twenty-second day, and it was suggested that such a rise might indicate a practical means of distinguishing pigs which have shed virus at parturition. After the second and third farrowings, in the absence of virus shedding, the serum neutralizing antibody titers rose only by insignificant amounts, supporting the previously expressed view. It must, however, be emphasized that the data are still too limited to make firm conclusions and to assume that pseudorabies virus infections differ in this respect from the better studied Herpes simplex infections.
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SECTION III. A PRELIMINARY REPORT ON THE USE OF HORMONAL SIMULATION OF PREGNANCY AND PARTURITION IN AN ATTEMPT TO RECRUDESCE LATENT PSEUDORABIES VIRUS IN PIGS
A preliminary report on the use of hormonal simulation of pregnancy and parturition in an attempt to recrudesce latent pseudorabies virus in pigs

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SUMMARY

An unsuccessful attempt was made to provoke the shedding of latent pseudorabies virus in female pigs by simulating pregnancy and parturition using hormones. The pro forma of the hormone injections is given and the possible reasons for the failure of the attempt are discussed.
Pseudorabies (PR, Aujeszky's Disease) virus has been shown by direct examination of various tissues to remain viable in the pig in a latent or persistent form for periods of at least 13 months (Nikitin, 1961; Sabó and Rajčáni, 1976; Beran et al., 1980) and part at least of its genome has been shown to remain for seven months (Gutekunst, 1979).

There is epidemiological evidence that the virus may be shed and infect other pigs under conditions of stress, including movement, farrowing and lactation (Nikitin, 1961; Howarth, 1969; Beran et al., 1976), and direct evidence that the virus may be shed in the nasal secretions of a postparturient sow, 19 months after clinical infection (Davies and Beran, 1980).

Latently infected pigs have been artificially induced to shed pseudorabies virus by the administration of dexamethasone, a synthetic cortisol (Crandell et al., 1979). Mice latently infected with another of the Herpetoviridae, Herpes simplex type 1, have been induced to shed virus by the administration of prostaglandins (Blyth et al., 1976). Both cortisol and prostaglandin are involved in normal parturition (First and Bosc, 1979).

In this report are described attempts to provoke the shedding of pseudorabies virus from previously infected and fully recovered non-pregnant gilts by hormonal simulation of pregnancy and parturition.
MATERIALS AND METHODS

The 5 pigs used in these experiments were pseudorabies-seronegative females of mixed breeding derived from a herd known and tested to be free of pseudorabies disease.

They were placed in isolation and infected when 5 weeks of age by instillation into each nostril of 100,000 plaque forming units of the 7th cell culture passage of strain 62/26 ("Io:xm" strain, "Wilson" strain) of virus, and they developed typical signs of pseudorabies. One pig died and the other 4 eventually made complete recoveries.

At 28 weeks of age, one pig was removed from the group and placed in single isolation where it was injected with Pregnant Mare Serum (PMS)\(^1\) and Human Chorionic Gonadotrophin (HCG)\(^2\) to induce estrus by the 4th day and ovulation by the 5th day. On the 17th day, it was injected with Estradiol Cypionate (ECP)\(^3\) to maintain the corpora lutea and thereby simulate pregnancy. On the 40th day, a regimen of injections of estradiol, adrenocorticotropic hormone (ACTH)\(^4\) and prostaglandin \(\text{F}_{2\alpha}\)

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\(^1\)Pregnant mare serum. PMS. Prepared in our laboratory as unrefined serum from 60-80 day pregnant mares. PMS was standardized in prepuberal mice (75 units resulting in the maximum average ovulation rate).


\(^3\)Estradiol Cypionate. ECP. The Upjohn Company, Kalamazoo, Michigan 49001.

\(^4\)Adrenocorticotropic hormone. ACTH. Adrenomone. Burns-Biotec Laboratories Division, Chromalloy Pharmaceutical Inc., Oakland, California 94621.
(PGF$_{2\alpha}$)$_1$ was started to induce pseudofarrowing on the 47th day (Table 1).

Table 1. Schedule of administration of drugs for all 4 pigs

<table>
<thead>
<tr>
<th>Event</th>
<th>Day</th>
<th>PMS (Units)</th>
<th>HCG (Units)</th>
<th>Estradiol (mg)</th>
<th>ACTH (Units)</th>
<th>PGF$_{2\alpha}$ (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>400 (600)$^a$</td>
<td>200 (300)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrus</td>
<td>4</td>
<td></td>
<td>(800)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovulation</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>17</td>
<td></td>
<td>10 (15)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td>3 (5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudo-</td>
<td>42</td>
<td></td>
<td></td>
<td></td>
<td>3 (5)</td>
<td></td>
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<tr>
<td>pregnancy</td>
<td>44</td>
<td></td>
<td>5 (7)</td>
<td>20 (20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td></td>
<td>5 (7)</td>
<td>40 (40)</td>
<td>10 (10)</td>
<td></td>
</tr>
<tr>
<td>Pseudo-farrowing</td>
<td>47</td>
<td></td>
<td>10 (14)</td>
<td>40 (40)</td>
<td>10 (10)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Doses marked in parentheses ( ) were given to the 40-week-old pigs. Unmarked doses were given to the 28-week-old pig.

The remaining 3 pigs were kept as a group and when they were 40 weeks of age they were treated to a similar regimen of injections, except that doses were increased to compensate for their greater size. Since they may at that age have been sexually active, they were given a

$^1$Prostaglandin F$_{2\alpha}$. PGF$_{2\alpha}$ Lutalyse (dinoprost tromethamine). The Upjohn Company, Kalamazoo, Michigan 49001.
further dose of HCG on the 4th day.

The schedule of administration of drugs for all 4 pigs is shown in Table 1. The doses used in the older pigs are distinguished by being marked in parentheses.

Nasal and oral swabblings were collected frequently before and during the period of pseudopregnancy, and daily for 2 weeks after the pseudofarrowing. These specimens were tested for the presence of pseudorabies virus by inoculating tube cultures of Madin-Darby bovine kidney (MDBK) cells. Cultures were incubated at 37°C for four days and observed daily for cytopathic effects (CPE) typical of pseudorabies virus.

Blood sera were collected at various times, and were tested for the presence of pseudorabies virus neutralizing antibodies against 300 tissue culture ID$_{50}$ of virus, using standard methods.

About 6 weeks after the pseudofarrowing, the pigs were slaughtered and the tonsils, turbinates, olfactory bulbs, optic nerves and trigeminal ganglia were tested for pseudorabies virus as follows: The test for active infection was by grinding parts of the tissues in buffered saline and placing the suspensions on MDBK cells and observing for CPE; the test for latent infection was by maintaining part of the tissue as intact fragments in nutrient medium and testing the supernatant fluids for released virus over the following 5 weeks (previously described by Beran et al., 1980).
RESULTS

The strain of pseudorabies virus used in these experiments caused severe clinical disease characteristic of pseudorabies in all of the 5 inoculated pigs. The most prominent signs of illness were pyrexia (to 41.6°C), rhinitis, sneezing, and, in some cases, incoordination. One of the pigs died and the other 4 eventually made complete clinical recoveries. Nasal secretions at the height of the infection contained an agent which caused CPE on MDBK cells and which was identified as pseudorabies virus by the neutralization test using pseudorabies-antisemum.

The regimen of hormone injections used to induce estrus and to simulate pregnancy and parturition had no apparent adverse effect upon the health of the pigs. Estrus was not observed during the periods of pseudopregnancy. Obvious mammary hyperplasia developed during the few days preceding the pseudofarrowing, and there was substantial enlargement and reddening of the vulva at the time of the pseudofarrowing. Neither vaginal discharge nor milk production was noted.

At no time prior to or during pseudopregnancy, or after pseudofarrowing, was virus detected in the pigs' nasal secretions or in their saliva, nor was virus detected in the tonsils, turbinates, olfactory bulbs, optic nerves or trigeminal ganglia collected about 6 weeks after pseudofarrowing. Both the direct examination for free virus in suspensions of the tissue placed on MDBK cell monolayers, and the examination of the supernatants of the tissue fragments maintained in nutrient media
for 5 weeks yielded negative results.

At 22 days after clinical pseudorabies, the serum neutralizing antibody titers of the convalescing pigs ranged from 1:4 to 1:16. The titers were essentially the same when tested again just prior to pseudopregnancy and 4 weeks after pseudofarrowing.
DISCUSSION

The experiments described were not successful in their main purpose, which was to demonstrate that hormonal simulation of pregnancy and parturition could provoke the shedding of pseudorabies virus in the nasal secretions of pigs which had been previously infected with that disease.

There are a number of possible reasons for this failure. In previous experiments (Beran et al., 1980), using the same strain of virus as inoculum, latent infection was demonstrated by recovery of virus from explants of tissues after 3 weeks - 13 months in 6 of 7 pigs tested. In the present study, virus could not be obtained from explants of the various tissues derived from the pigs after slaughter, when tested both for free infectious virus and for latent virus. There is, therefore, a strong possibility that latent infection was not established in these pigs. Why this group of pigs should be different from the previous group is not known. The possibility remains that the pro forma of hormone injections described might have been perfectly successful if it had been used to recrudesce virus shedding in pigs actually latently infected.

It is also possible that the hormone treatment given did not realistically simulate the changes which led to the shedding of virus in nasal secretions for a 6-day period following natural parturition in one sow studied in this laboratory (Davies and Beran, 1980). Although the hormone treated gilts were anestric during the pseudopregnant period
and mammary hyperplasia and vulvar enlargement were observed at the time of pseudofarrowing, there can be no assurance that the complex changes of natural parturition were adequately simulated.

The changes which led to shedding in the naturally parturient sow may have included stresses in addition to the hormonal changes from pregnancy. The sow was inadvertently stressed in the immediate postparturient period by a failure in the water supply and needed subsequent medication with gentamycin sulfate and oxytocin. The pseudopregnant gilts were subjected to no recognized environmental stresses during the period of hormone injections.

The inability of the tested procedure to cause recrudescent shedding of pseudorabies virus in convalescent gilts through hormonally induced pseudopregnancy and pseudofarrowing would have been much more conclusive if latent infection could have been demonstrated in these animals when examined at necropsy. Further studies are definitely needed until it can be demonstrated whether or not latently-infected convalescent gilts can be induced to shed virus through manipulation of hormonal activities.
REFERENCES


ACKNOWLEDGMENTS

The authors thank Dr. H. T. Hill and his laboratory for performing serum neutralization tests, and Dorothy Murphy, Charles Gold and Virginia Smith for technical assistance.

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SECTION IV. CHARACTERISTICS OF A STRAIN OF PSEUDORABIES VIRUS OF LOW VIRULENCE NEWLY ISOLATED IN IOWA, AND COMPARISON WITH A PREVIOUSLY KNOWN STRAIN OF HIGH VIRULENCE
Characteristics of a strain of pseudorabies virus of low virulence newly isolated in Iowa, and comparison with a previously known strain of high virulence

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Supported by a State of Iowa Livestock Health Advisory Council Grant, and the United States Department of Agriculture Science and Education Administration, National Animal Disease Center, Ames, Iowa 50010.
A strain of pseudorabies virus (PRV) of low virulence, designated "IA80" strain, was isolated from the brain of a 7-day-old pig which had shown incoordination since birth. The pig derived from a herd which had shown no clinical signs of pseudorabies virus, which had a normal conception and farrowing rate, and from which virus had not been isolated previously despite an extensive search on account of periodic seroconversions.

The pathogenicity and immunogenicity of the IA80 strain of virus are compared with those of the S62/26 strain of virus, and its possible significance in disease eradication programs discussed.
The eradication of any infectious disease in the field depends upon the easy and unequivocal identification of the disease by recognition of clinical signs, by isolation of the infectious agent, or by detection of changes in the immunological status of animals affected.

In previous years, the prevalent strain or strains of pseudorabies virus in Iowa have caused relatively distinctive clinical signs, particularly in young pigs, and the virus has usually been isolated without great difficulty for positive identification in the laboratory by serum neutralization or fluorescent antibody tests. Recovered animals have shown the production of serum neutralizing (SN) antibodies, and these have been used to identify previous infections, and freedom from SN antibodies has been required by law for interstate transport of pigs to breeding and finishing units.

This report discusses the isolation and the characterization of a strain of pseudorabies virus from a herd of pigs in Iowa in which at no time were there outbreaks of clinical disease suspected of being pseudorabies, and from which virus had not been isolated in recent years, despite continuing seroconversions.
MATERIALS AND METHODS

The herd of pigs used in this study was a closed farrow to finish operation in northern Iowa with 300 sows and a normal inventory of 3,000 head. The conception rate was over 90%, and sows farrowed over 9 pigs per litter and weaned over 8 pigs per litter twice a year. Feed conversion from weaning to market was 2.7 lbs per lb of gain. Gilts were routinely vaccinated with a modified live pseudorabies virus vaccine\(^1\) before breeding, and in the last year were revaccinated two weeks before farrowing. Pseudorabies virus had been isolated in the herd from only one pig which was not observed to have any clinical signs other than diarrhea in 1976, but not since, despite numerous attempts by nasal swabs and testing of tissues at necropsy in various laboratories. Numerous seroconversions, however, had been observed in fattening pigs since this isolation and had had a seriously detrimental effect upon the sale of pigs.

A seven-day-old pig which from birth had shown signs of incoordination was submitted to the laboratory from this herd for diagnosis of a coexisting diarrhea. It was killed and necropsied and a sample of its brain was tested for pseudorabies virus by emulsifying it in buffered saline, centrifuging the suspension and adsorbing the supernatant on Madin Darby Bovine Kidney (MDBK) cells in tubes for one hour. The brain suspension was then replaced with Minimum Essential Medium (MEM)\(^2\) + 1% 

\(^1\)PR-Vac. Norden Laboratories, Inc., Lincoln, Nebraska 685501.
\(^2\)Grand Island Biological Company, Grand Island, N.Y. 14072.
Fetal Bovine Serum (FBS) + 1% new born calf serum (NCS) + antibiotics; the tubes were incubated at 37°C in 5% carbon dioxide atmosphere and observed daily for cytopathic effect (CPE). In due course, the identity of the agent which caused CPE was confirmed as PRV by neutralizing it with specific antiserum. As it was isolated in northern Iowa in 1980, it was named "IA80 strain" pseudorabies virus.

The pathogenicity and immunogenicity of the IA80 strain of virus were tested in pigs in two experiments.

In the first experiment, 6 pigs aged 1 month of mixed breeding from a herd known and tested to be free of pseudorabies virus were individually serologically tested for the presence of pseudorabies virus neutralizing antibodies against 300 plaque forming units (PFU) of virus using standard methods. Two of the pigs were held in reserve in isolation. The other four were placed in isolation and inoculated with 0.5 ml of IA80 virus, first passage in MDBK, in each nostril, to give a total of 10,000 PFU of virus per pig. On the 3rd day, one pig was killed and necropsied, and its olfactory bulb, optic nerve, trigeminal ganglia, cerebrum, cerebellum, medulla oblongata, cervical spinal cord, turbinates, tonsils, retropharyngeal lymph nodes, lungs, spleen, liver, kidneys, urinary bladder and jejunum were examined by histopathological methods and also assayed for virus by emulsifying and placing on MDBK in a manner similar to that used previously to isolate the virus. On the same day, the 2 remaining uninfected pigs were added to the infected group of pigs as incontact sentinels. On the 9th day, another of the intranasally

1Penicillin G potassium, streptomycin sulfate, amphotericin B, and gentamicin sulfate.
infected pigs was killed and necropsied, and the same list of tissues was examined by histopathological and virological methods. The pigs were clinically examined and their temperatures monitored daily for 2 weeks. Their sera were tested for titers of PRV neutralizing antibody on the 31st day and on the 58th day (28th and 55th days for sentinels). On this latter day, the 2 surviving intranasally-infected and the 2 sentinel pigs were killed and necropsied, and the same list of tissues was again examined by virological methods. In addition, small portions of the olfactory bulb, optic nerves, trigeminal ganglia, turbinates and tonsils were maintained in 3 ml of MEM + 1% FBS + 9% NCS + 0.25% lactalbumin hydrolysate + antibiotics as previously described (Beran et al., 1980) and tested for shedding of latent virus by placing aliquots of the supernatant fluid on MDBK test cells at weekly intervals for 5 weeks.

In the second experiment, 18 pigs aged 1 month, of mixed breeding from another herd known and tested to be free of pseudorabies virus were randomized and individually serologically tested for the presence of pseudorabies neutralizing antibodies as described earlier. Four pigs were placed in each of 3 isolation pens, and the remaining 6 pigs were held in reserve in separate isolation. The pigs in the first group were each inoculated with 0.5 ml of IA80 virus, second passage in MDBK, in each nostril, to give a total of 100,000 PFU of virus per pigs. The pigs in the second group were each inoculated with 100,000 PFU of the same virus intramuscularly in one site in the gluteal muscles of the right hind leg. The pigs in the third group were each inoculated with
0.5 ml of S62/26 ("Iowa strain," "Wilson strain") virus, ninth passage in MDBK, in each nostril, to give a total of 100,000 PFU of virus per pig. On the 3rd day, one pig from each group was killed and necropsied and tissues examined by histopathological and virological methods, as in the first experiment. The blood was also tested for the presence of virus. On the same day, 2 of the pigs held in reserve were added to each group of infected pigs as incontact sentinels. On the 6th day, the 3 remaining pigs which had been inoculated intranasally with S62/26 virus were dead or were killed in extremis and from each, the jejunum, rectum, kidneys and urinary bladder were collected for virological examination. On the 7th day, another of the originally inoculated pigs was killed in each of the groups given IA80 virus and tissues taken for histopathological and virological examination. All pigs were clinically examined and their temperatures were recorded daily for 18 days. Their sera were tested for pseudorabies neutralizing antibody as previously described on the 21st, 31st and 51st days (18th, 28th and 48th days for the sentinels). The sera of the 2 pigs which had been placed in contact with the pigs infected intramuscularly with IA80 virus were also tested on the 69th day.
RESULTS

The agent which was isolated from the brain of the 7-day-old pig caused cytopathic effect typical of pseudorabies virus in MDBK cells and was neutralized by specific pseudorabies antiserum.

In the first pig experiment, when 10,000 PFU of the IA80 virus were inoculated intranasally into young pigs, there were no clinical signs or pyrexia in these animals or in the sentinels put in contact with them. The temperature of all the pigs remained below 39.6°C. Virus was detected in the turbinates, tonsils, retropharyngeal lymph nodes, lungs and spleen of the pig killed on the 3rd day, and in the tonsils of the pig killed on the 9th day. Histology of the pig killed on the 3rd day showed no pseudorabies virus-related lesions except for one focal infiltration of the trigeminal ganglia with lymphocytes and macrophages. In the pig killed on the 9th day, the microscopic lesions included mild perivascular cuffing and some glial nodules adjacent to the floor of the 4th ventricle in the medulla oblongata and a tonsillar crypt which had epithelial necrosis. The serum neutralizing antibody titers of the 2 remaining pigs which had been inoculated intranasally were 1:16 and 1:32 on the 31st day and 1:8 and 1:16 on the 58th day. The serum neutralizing antibody titers of the 2 sentinel pigs were 1:64 and 1:32 on the 28th day and 1:8 each on the 55th day after being placed in contact. Virus was not detected in any of the tissues of the pigs when killed and tested on the 58th day (55th day for sentinels), either as free or latent infection.

In the second pig experiment, when 100,000 PFU of the IA80 virus
were inoculated intranasally into young pigs, they rapidly developed a serous virus-containing nasal discharge which lasted from the 3rd to the 9th day, and a transient pyrexia to 41.4°C on the 3rd day. Virus was detected in the olfactory bulbs, optic nerves, trigeminal ganglia, medulla oblongata, turbinates, tonsils, retropharyngeal lymph nodes and blood of the pig sacrificed on the 3rd day. The only histological lesions observed were scattered glial foci in the brain stem. One pig became seriously ill beginning on the 6th day and was killed on the 7th day when incoordinated and lying on its side making paddling motions with its legs. Virus was recovered from its optic nerves, trigeminal ganglia, medulla oblongata, turbinates and tonsils. In this animal, histology revealed glial foci and perivascular cuffing in the cerebrum, cerebellum and brain stem, and suppurative lymphadenitis of the retropharyngeal lymph node. The other intranasally infected pigs showed no clinical signs other than the serous nasal discharge, even at the height of the pyrexia. Their serum neutralizing antibody titers were 1:8 and 1:16, respectively, on the 21st day and were unchanged on the 31st and 51st days. The 2 sentinel pigs developed slight serous nasal discharges on the 4th through 6th days after being placed in contact with the intranasally infected pigs, and pseudorabies virus was isolated in the nasal discharges of one of the pigs on the 6th day. Neither sentinel pig showed any abnormal clinical signs or pyrexia. Their serum neutralizing antibody titers were 1:16 and 1:8 on the 18th day of contact, 1:32 and 1:4 on the 28th day and 1:16 and 1:8 on the 48th day.

When 100,000 PFU of the IA80 virus were inoculated intramuscularly
into young pigs, they did not develop pyrexia, the temperature of all pigs remaining below 40.1°C. The only clinical sign was that one pig developed sciatic paralysis in the leg which was injected. Virus was detected in the olfactory bulbs, cervical spinal cord and tonsils of the pig killed on the 3rd day, and in the optic nerves of the pig killed on the 7th day. No lesions were noted histologically in the first of these pigs, while in the second there were glial foci and perivascular cuffing in the cerebrum, cerebellum and brain stem. Although there was no nasal discharge in any of the pigs, virus was recovered from nasal swabs taken from one of the pigs on days 5 through 7, 9 and 14. The serum neutralizing antibody titer of the pig which shed virus was 1:4 on each of days 21, 31 and 51. The titer of the neutralizing antibody of the pig which did not shed virus was 1:8 on each of the same days. The two pigs which were used as incontact sentinels did not show pyrexia, nasal discharge or any other clinical signs. In one of these pigs, neutralizing antibodies of titer 1:4 were detected on the 48th and 69th days after contact with the inoculated pigs. No neutralizing antibodies to pseudorabies virus were detected in the serum of the other incontact pig.

The young pigs which were inoculated intranasally with 100,000 PFU of S62/26 virus were severely affected. By the 3rd day, 3 pigs had temperatures in excess of 41.0°C, and the 4th pig had a temperature of 41.7°C. One pig was killed on the morning of the 3rd day and virus was recovered from its olfactory bulbs, optic nerves, trigeminal ganglia, cerebrum, medulla oblongata, cervical spinal cord, turbinates, tonsils,
and retropharyngeal lymph nodes. Histology revealed small glial foci in its brain stem and necrotic foci in its tonsilar crypts. The other 3 pigs all developed severe purulent nasal discharges by the 4th day, followed rapidly by panting, snuffling breathing and lethargy. By the morning of the 6th day, one pig was dead and the other 2 were recumbent, dehydrated, cyanotic, and showed muscular tremors, and had falling temperatures. A second pig died during the day and the third was killed while in extremis. Virus was recovered from the kidneys of one of these 3 pigs. It was not recovered from the urinary bladder, jejunum or rectum of any of them. Virus was recovered regularly from the nasal discharges of all 4 pigs from the first day until the time of death. One of the 2 pigs used as incontact sentinels developed serious clinical signs of pseudorabies. It had a purulent nasal discharge from the 2nd through the 10th day and a temperature of 41.6°C by the 7th day. By the 8th day the animal was incoordinated, and on the 9th it was recumbent and making paddling motions with its legs. When held to the drinking bowl, it swallowed with difficulty. On the 10th day, it could stand stiff-legged but it could find the water bowl. Recovery thereafter was slow with the animal showing severe nervous dysfunction for several more days, until after another 10 days it appeared as a runted and stiff-legged but otherwise recovered pig. Despite the prominent purulent nasal discharge, virus was only recovered from the nasal secretions on the 5th and 7th days. The other incontact sentinel pig developed only mild clinical disease. It showed no other clinical signs than a purulent nasal discharge on the 3rd day only. Its temperature did not rise about 40.8°C. The
serum neutralizing antibody titer of the first of the incontact sentinel pigs was 1:8 on each of days 18, 28 and 48. The titers for the other sentinel pig were 1:8, 1:8 and 1:32 on the same days.
DISCUSSION

The isolation of the IA80 strain of virus from the brain of a pig which was only 7 days old and which had shown incoordination from birth suggested that infection had been acquired in utero. This supposition is supported by the fact that experimentally the virus was well-transported around the bodies of young pigs when inoculated either intramuscularly or intranasally. Despite its presumed transplacental spread, and despite the numerous seroconversions in the herd from which the index case originated, the virus had not upset the conception rate or caused abortions. In this, it differs from the S62/26 strain, which was also shown to cross the placenta (Hill and Maré, 1975), causing abortions and deaths of newborn pigs.

The characteristics of the IA80 virus when experimentally inoculated into susceptible pigs were unlike those of the modified live virus vaccine which had been administered to the gravid sow, particularly in its ability to spread around the body further than the regional lymph nodes, its causing sciatic paralysis, and its being shed from the nostrils.

When a large dose of the IA80 strain of virus was administered intranasally, it caused serious illness in only one of the 12 pigs tested (taking the two experiments together and including the sentinel pigs). The virus was invasive and was recovered from several tissues including central nervous system tissues distant from the site of inoculation as well as from the blood. It was shed for up to 9 days, it spread to sentinels and it was quite immunogenic. In a field situation, it is
possible that it would spread from pig to pig without being recognized and that it would cause seroconversion of the infected pigs.

When the IA80 strain of virus was administered intramuscularly, it caused sciatic paralysis in one of the 4 pigs tested, but no other clinical signs. Sciatic paralysis was recorded with an Iowa field strain in 1935 by Shope, but is not a feature of the modified live virus vaccine which was used in this herd. As when it was administered by the intranasal route, the IA80 virus was again observed to be invasive, and was recovered from several tissues around the body including from the central nervous system and was observed to be shed intermittently from the nostrils of one pig up until the 14th day. The sentinel pig which seroconverted did so some time between the 28th and 48th days, again indicating late shedding of virus, perhaps even after that noted by nasal swabbing. The surprising length of this period of shedding may have been due to the rather low titer of circulating serum neutralizing antibody produced by this pig (1:4 on the 21st day) or because the route of infection did not encourage the production of a local immune response. Although virus was not recovered from the nasal secretions of the pig which was killed on the 3rd day, it was recovered from the olfactory bulbs and the tonsils, both sites from which there would presumably have been the potential for shedding. In view of the well-known propensity for certain of the Herpetoviridae to sequester themselves in nervous tissue and to be shed to the epithelial surface down the axons of nerves, it is possible that the nasal shedding observed was via the olfactory bulb route, rather than direct hematogenous spread to the nasal epithelium. It may also be
suggested that in view of the presumed transplacental infection of the index case, the ease with which the virus may spread around the body of young pigs, and its observed shedding for long periods from the nostrils of the young pig when it reached those tissues centrifugally, carrier sows could transmit the virus to their fetuses. In turn, they could then shed it from their nostrils after birth, thereby infecting other pigs. This would be in addition to the possibility of direct spread by nasal shedding from the sow in the postparturient period, as has already been described (Davies and Beran, 1980).

As expected from previous work (Beran, unpublished), the S62/26 strain of virus was highly pathogenic for young pigs, producing classic symptoms and (taking inoculated pigs and sentinels together), severe illness or death in 5 of 6 pigs. The sample of virus used was the 9th passage in MDBK cell culture, which was the lowest passage number available. The passaging in this cell culture did not, however, appear to have noticeably modified its pathogenicity in pigs as compared to the pathogenicity of the freshly isolated field virus. The isolation of virus from the kidneys of the pig killed in extremis raises the possibility of the spread of pseudorabies virus in urine, as well as by aerosols and nasal secretions. Virus has been previously reported by European workers in kidneys and urine (Review by Baskerville et al., 1973). If virus is shed in urine, it may survive quite well (Freund and Beran, ca. 1981).

A comparison of the virological and histopathological testing of tissues at necropsy showed that at the times tested, the former technique
was the more sensitive means of detecting the presence of pseudorabies virus. With the low dose of IA80 virus, no lesions typical of pseudorabies could be detected in the brain by histopathological examination, while with the very large dose there were some mild lesions of non-suppurative encephalitis.

The IA80 strain of virus is of some interest in pseudorabies eradication programs. Prior to its isolation, the herd of pigs from which it originated had been plagued with unexplained seroconversions. As there was no clinical illness, as conception and farrowing rates were normal, and as pseudorabies virus could not be isolated from the herd, speculation had arisen that a serologically cross-reacting infectious agent related or unrelated to the Herpetoviridae might be present in the herd, interfering with the test and giving false positive results (Beran and Davies, 1981). The eventual isolation of the IA80 strain of virus provides a much more likely explanation of the continuing seroconversions, and reinforces the currently held view that there is no agent which elicits antibodies in pigs which cross-reacts with PRV.
REFERENCES


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The authors thank the owner of the herd of pigs in northern Iowa for his full cooperation in this investigation, Dr. H. T. Hill and his laboratory for performing serum neutralization tests, and Dorothy Murphy, Charles Gold and Virginia Smith for technical assistance.

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SECTION V. ESTABLISHMENT OF PERSISTENT PSEUDORABIES VIRUS INFECTION IN PORCINE TURBINATE, SALIVARY GLAND AND TONSILAR FRAGMENTS MAINTAINED IN VITRO
Establishment of persistent pseudorabies virus infection in porcine turbinate, salivary gland and tonsilar fragments maintained in vitro

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Supported by a State of Iowa Livestock Health Advisory Grant, and the United States Department of Agriculture Science and Education Administration, National Animal Disease Center, Ames, Iowa 50010.
Persistent productive pseudorabies infections were established in porcine turbinate, salivary gland and tonsilar fragments maintained in vitro for up to 126, 56, and 51 days, respectively. The addition of serum neutralizing antibodies to the system for prolonged periods did not clear the infection of the turbinate and salivary gland fragments. When the antibodies were removed, infectious virus was shed and detected up until the 189th and 182nd days, respectively, after initial infection. Virus was never recovered from the supernatant fluids of tonsilar tissues maintained in the presence of neutralizing antibodies.
INTRODUCTION

Many of the Herpetoviridae are able to establish latent infections in their host with periodic episodes of recurrent clinical disease (Fenner et al., 1974). The mechanisms by which this occurs have been the subject of extensive investigation, but are still poorly understood.

Latent infection with pseudorabies (Porcine herpesvirus I, Aujeszky's Disease) virus has been shown to occur in pigs by testing of their tissues for virus for periods of up to 13 months after clinical disease (Nikitin, 1961; Sabó and Rajčáni, 1976; Beran et al., 1980), by RNA-DNA hybridization for up to 7 months (Gutekunst, 1979), by shedding of virus after cortisol treatment for up to 3 months (Crandell et al., 1979), and by shedding from the nostrils of a sow in the postparturient period 19 months after initial infection (Davies and Beran, 1980).

An understanding of the mechanism of the establishment and maintenance of latency is of great theoretical interest and practical importance, but the maintenance of pigs in isolation for the long periods necessarily required in these experiments is expensive, and the whole animal is a complex interrelationship of different tissues and immunological responses. It would be highly advantageous if the phenomenon of latency could be simulated in isolated tissue fragments maintained in the laboratory.

Persistent infections of cell cultures have been established using many families of viruses mainly in continuous cell lines (Reviews by Huang and Baltimore, 1970; Stevens et al., 1978), and various mechanisms
of persistence have been proposed, such as defective interfering particles (Huang and Baltimore, 1970), temperature sensitive particles (Preble and Youngner, 1975), and integration in the cellular genome using reverse transcriptase (Simpson and Inuma, 1975; Zhdanov, 1975). Whether these laboratory models of persistence are in any way related to latency in the whole animal is not yet clear.

This report describes attempts to establish persistent infection in tissue fragments containing the natural mixed population of cells maintained in vitro, both in the presence of and in the absence of specific neutralizing antibodies.
MATERIALS AND METHODS

The tissue fragments used in the two experiments described here were derived from pigs bred and reared in a herd known and tested to be free of pseudorabies virus infection. Animals in the herd were not vaccinated against pseudorabies.

At one month of age, animals were stunned by electricity and then slaughtered by exsanguination. Their turbinates, salivary glands and tonsils were collected aseptically within a few minutes of death and held as portions of less than 1 cm in diameter in physiological saline-glucose solution (saline G) containing antibiotics\(^1\) in Petri dishes at room temperature for not longer than 1 hour before further processing. The tissues were then cut into fragments approximately 1 mm in diameter and 6-8 of them were placed in 2.6 ml of Minimum Essential Medium (MEM)\(^2\) containing antibiotics\(^1\) in plastic tubes\(^3\) of size 16 x 125 mm with loosely fitting caps. Similar tubes were set up without tissue fragments as controls.

The virus used in the experiments was the 62/26 strain, passaged 9 times in Madin Darby Bovine Kidney (MDBK) cell cultures. Some of the tubes containing tissue fragments and some of the tubes which did not contain tissue fragments were inoculated with approximately 10,000

\(^1\)Penicillin G Potassium, streptomycin sulfate, amphotericin B, and gentamicin sulfate.

\(^2\)Grand Island Biological Company, Grand Island, N.Y. 14072.

\(^3\)Falcon, 1950 Williams Drive, Oxnard, CA. 93030.
Plaque Forming Units (PFU) of virus contained in 0.1 ml of suspension. The actual number of PFU added to the tubes was then confirmed by making serial dilutions of another aliquot of this virus suspension in saline G and testing its ability to form plaques on MDBK monolayers, using standard methods (Davies, 1979).

After inoculation of virus suspension, all tubes were incubated at 37°C for 1 hour, and then 0.3 ml of porcine serum was added to each. The serum added was derived either from nonimmune pigs aged 1-3 months or from a pseudorabies-immune pig aged 3 months which had been infected when 1 month old with pseudorabies strain 62/26 passage 7 virus. The immune serum had a serum neutralizing titer of 1:8 when tested against 300 PFU of pseudorabies virus using standard microtitration methods. The non-immune serum had no detectable serum neutralizing antibodies. A third set of tubes was inoculated with a dilution containing one part of the immune serum with 4 parts of the nonimmune serum. None of the sera was treated by heating or in any other way.

After addition of sera, all tubes were incubated at 37°C for 1 hour. An aliquot of 0.2 ml of supernatant fluid was then removed, serial dilutions were made in saline G and titrated by testing for ability to form plaques on MDBK monolayers, as previously described.

For long-term storage, all tubes were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide. On the 3rd or 4th and the 7th days, and then subsequently at intervals of 1 or 2 weeks, small samples of the supernatant fluid were removed from each tube and assayed for virus, again using the plaque counting technique. In addition, from
the 7th day onward further samples were tested for the presence or absence of virus by inoculation onto MDBK cell monolayers grown in tubes. After adsorption for 1 hour, the test solutions were removed, the cells were washed with MEM, and then nutrient medium (MEM + 1% Fetal Bovine Serum + 1% Newborn Calf Serum) was added. Cultures were incubated and the cells were observed for cytopathic effect (CPE) during each of 4 days. The identity of agents causing CPE was confirmed by further passaging and testing with specific pseudorabies-antiserum.

From the 7th day onward, after the sampling of the supernatant fluids as described above, the remainder of the supernatant fluid was removed from each tube containing tissue fragments, but not from the tubes with no fragments, and replaced with fresh MEM with antibiotics and the appropriate immune or nonimmune serum for that tube. In some instances, the removed tissue-free supernatants were then placed in capped plastic tubes and maintained in the incubator alongside the tissue-containing tubes from which they derived. Samples of these fluids also were occasionally tested for free virus over the next several weeks by observation of their ability to cause CPE in MDBK tubes.

When virus had not been detected for several weeks (see Figures 1 and 2) in any of the group of tubes containing one type of tissue, the antibody-containing serum was replaced with nonimmune serum, and the monitoring was continued to see if virus was subsequently released from the tissue fragments.

In the second experiment, the viability of the tissue fragments was estimated at the end of the experiment by histological examination, and
Figure 1. Experiment 1 - Titer of virus in supernatant fluid of tubes containing turbinate, salivary gland, tonsilar and no tissue fragments
Tubinate Tissue Fragments

- Amount of Viral Added
- Viral Titre
- Antisera replaced by Non-Immune Serum
- Virus detected by tube test
- Virus not detected by tube test

Non-Immune Serum + Virus
Low Level Anti-Serum + Virus
High Level Anti-Serum + Virus
Non-Immune Serum No Virus

Salivary Gland Tissue Fragments

Non-Immune Serum + Virus
Low Level Anti-Serum + Virus
High Level Anti-Serum + Virus
Non-Immune Serum No Virus
Figure 2. Experiment 2 - Titer of virus in supernatant fluid of tubes containing turbinate, salivary gland, tonsilar and no tissue fragments
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Tube No. 1: Turbinate Tissue Fragments
Tube No. 2: Tonsil Tissue Fragments
Tube No. 3: Salivary Gland Tissue Fragments
Tube No. 4: No Tissue Fragments

Amount of Virus Added
Virus Titer
Amount of Virus less than 10
Amount of Virus greater than 10

Antibody Activity

- Virus detected by tube test
- Virus not detected by tube test

Non-Immune Serum + Virus
Low Level Anti-Serum + Virus
High Level Anti-Serum + Virus
Non-Immune Serum No Virus
Non-Immune Serum + Virus
Low Level Anti-Serum + Virus
High Level Anti-Serum + Virus
Non-Immune Serum No Virus
by challenge of the tissue fragments with approximately 1,000 PFU of pseudorabies virus, followed by monitoring of the titer of the virus in the supernatant fluids to see if there was viral replication.
RESULTS

At no time was virus detected in the supernatant fluids of any of the porcine tissues maintained in culture without the addition of virus.

Experiment 1. Figure 1. The confirmatory titration of the stock virus suspension indicated that there were 16,600 FFU in the 0.1 ml added to the tubes, giving, therefore, before any interaction a theoretical titer in the supernatant fluids of approximately 5,500 FFU/ml.

Virus maintained in the absence of tissue fragments and antiserum (tube 13) was rapidly inactivated and none was detected after the 3rd day. In the absence of tissue fragments, but in the presence of the low or high levels of antiserum (tubes 14 and 15) the inoculated virus was not detected after 1 hour of interaction.

When virus was added to turbinate tissue fragments in the absence of antiserum (tube 1), a sustained high titer of virus was observed in the supernatant fluids for a considerable period of time, eventually disappearing after the 126th day. In the equivalent tubes (2 and 3) with low or high levels of antiserum, infectious virus was detected intermittently until the 112th day. When the antiserum was replaced with non-immune serum in these tubes on the 154th day, virus was not detected in the supernatant fluids subsequently.

When virus was added to salivary gland tissue fragments in the absence of antiserum (tube 5), a high titer of virus was observed in the supernatant fluids for several weeks. Virus was not recovered on the 42nd day, but reappeared at low titers on the 51st day and 56th days. In
the equivalent tube (7) with high levels of antiserum, virus was detected in the supernatant fluids on the 28th and 51st days, and after the antiserum was replaced with nonimmune serum on the 77th day, there was a sustained rise in titer of detectable virus which declined and disappeared after the 119th day. In contrast, in the tube (6) which received the low level of antiserum initially, virus was not detected at any time.

When virus was added to tonsilar tissue fragments in the absence of antiserum (tube 9), a high titer of virus was observed in the supernatant fluids for several weeks, eventually disappearing after the 51st day. In contrast, in the tubes (10 and 11) which initially received low or high levels of antiserum, virus was not detected at any time.

Experiment 2. Figure 2. The confirmatory titration of the titer of the stock virus suspension indicated that there were 17,300 PFU in the 0.1 ml added to the tubes, giving, therefore, before interaction a theoretical titer in the supernatant fluid of approximately 5,800 PFU/ml.

Virus maintained in the absence of tissue fragments and antiserum (tube 13) was rapidly inactivated and was no longer detected on the 4th day. In the absence of tissue fragments but in the presence of the low or high levels of antiserum (tubes 14 and 15) virus was not detected after 1 hour of interaction.

When virus was added to turbinate tissue fragments in the absence of antiserum (tube 1), virus was recovered consistently but with rapidly declining titer until the 43rd day, could not be recovered on the 49th and 56th day, but reappeared at quite high titer on the 63rd day and could still be detected on the 77th day. In the presence of low levels
of antiserum (tube 2), the titer of the virus in the supernatant fluid followed a very similar pattern of disappearance by day 56 and reappear­ance in quite high titer on day 77, before becoming undetectable after the 112th day. After the antiserum was replaced with nonimmune serum on the 182nd day, virus was again detected transiently and at low titer on the 189th day. In contrast, in the tube (3) which received the high level of antiserum initially, virus was not detected at any time.

When virus was added to salivary gland tissue fragments in the absence of antiserum (tube 5), the titer of the virus in the supernatant fluids declined to a very low level by the 35th day, but then increased abruptly to a high level on the 43rd day, before declining and disappearing after the 49th day. In the presence of low level of antiserum (tube 6), virus was recovered regularly at moderately high titer before declining and disappearing after the 63rd day. When the antiserum was re­placed with nonimmune serum on the 112th day, virus was again detected consistently at a moderately high level before declining. The last iso­lation of virus was on the 182nd day. In contrast, in the tube (7) which received the high level of antiserum initially, virus was not de­tected at any time.

When virus was added to tonsilar tissue fragments in the absence of antiserum (tube 9), a high level of virus was detected in the super­natant fluid until the 7th day only, when it disappeared abruptly. In the tubes (10 and 11) which initially received low or high levels of anti­serum, virus was not detected at any time.

Virus was not detected at any time in the tissue-free supernatant
fluids which were periodically collected and held at 37°C alongside the parent tubes containing tissue fragments from which they derived. Histological examination of samples of the tissue fragments used in the second experiment after 214 days indicated that in most of the tubes all the cells were dead. A few cells which were probably still alive were noted in tubes 2 and 4, containing turbinate tissue with virus plus low level antiserum, and turbinate tissue with neither virus nor antiserum, respectively. After challenge of the remaining portions of the tissue fragments with virus on the 215th day, the virus titer increased in the supernatant fluids of tube 3 (turbinate infected with virus in the presence of high level of antiserum).
DISCUSSION

Virus was not detected in the supernatant fluids of any of the uninfected control tissue fragments, indicating that the virus recovered in these experiments derived entirely from that which was added to the system, not from a previous latent infection of the pigs.

Previous experiments (Davies and Beran, ca. 1981) indicated that when this strain of pseudorabies virus was maintained at 37°C in a similar medium, it was inactivated at a rate of 0.6 log_{10} per day. In these experiments, virus maintained in the absence of tissue fragments was totally inactivated within several days, and the periodic changing of the medium served on each occasion to remove over 90% of virus in the supernatant fluids. Virus was never recovered from the supernatant fluids removed from tissue fragments and then stored in tubes alongside the fragment-containing tubes from which they derived, indicating that inactivated virions did not recover their infectivity with time, and that virion-antibody complexes did not disassociate to release infectious virus. Taken together, these factors indicated that when virus was detected in the supernatant fluid of a tube, it had been released from the tissue fragments within the previous several days.

In cell culture systems, such as MDBK cell monolayers, pseudorabies virus multiplies rapidly and infects and destroys all available cells. The titer of the virus in the supernatant fluids rises rapidly and then declines due to thermal inactivation as it is no longer replenished with freshly-produced virus. The peak titer is produced within 2 or 3 days,
and then declines regularly at a rate of around $0.6 \log_{10}$ per day. In
the first experiment described here, when virus was added to turbinate
tissue in the absence of antibodies, a titer of virus was maintained in
the supernatant fluid in excess of 1,000 PFU/ml for the first 106 days,
and virus did not disappear completely until after the 126th day, indic­
cating that an entirely different cell-virus mechanism was operating.
It is interesting to note that the titer was stable throughout the ex­
periment. This was also noted in another experiment not described here.
The mechanism for this phenomenon is not known. Virus was not tested
for changing antigenicity, temperature sensitivity, or the presence of
defective interfering particles. All that can be said is that the fre­
quent changing of the medium would preclude a feed-back mechanism de­
pendent upon high levels of some interfering substance or particles in
the supernatant fluids. In the second experiment, the virus titer was
not stable, but appeared to cycle, and at one stage, virus could not be
detected. Whether the virus during this period was entirely cell asso­
ciated in an integrated or nonintegrated form, or whether it was still
free virus at such a low titer that our sampling technique of taking
1/15th of the supernatant did not detect it, is not known. Salivary
gland and tonsil tissue fragments were not as effective in maintaining
virus infection as turbinate tissue fragments, but virus was still de­
tected in their supernatant fluids for up to 56 and 51 days, respectively.
It is interesting to note that in vivo, Burrows (1966) found that cattle
turbinate tissue was amongst the least effective of a wide range of
tissues in harboring one of the Picornaviridae, foot and mouth disease
The effect of the addition of antiserum to the system was of some interest. The maximum period of recovery of virus was increased with turbinate tissue from 126 to 189 days, and with salivary gland tissue from 56 to 182 days. While our in vitro system lacked many of the in vivo immunological components and so cannot be used to predict what would have happened in the whole animal, it was apparent that neutralizing antibodies alone cannot clear pseudorabies virus infection in the tissues. On 2 of the 4 occasions described here, once with high (Experiment 1) and once with low (Experiment 2) levels of antiserum, a sustained production of virus was noted from the salivary gland tissue fragments when the antisera was removed, at a time long after production of virus had ceased in the equivalent tube which had not been treated with antiserum. It is not clear at this time whether the virus had been driven by the antiserum into a nonreplicating cell-associated form, or whether replication continued normally and those virions which were released into the supernatant fluid were neutralized, leaving only a smouldering cell to cell infection.

Porcine turbinate tissue fragments were kept alive in vitro in this simple and economical system in excess of 200 days. Unlike continuous cell lines, these fragments contained a variety of cells and they were not transformed and so were closer to the tissues found in vivo. Persistent pseudorabies virus infections were regularly established in certain of these tissues as described. As far as is known, pseudorabies virus is a typical member of the Herpetoviridae and has little or no
known pathogenicity for human beings (Baskerville and McFerran, 1980), so it may properly be used as a safe laboratory tool. While it cannot at this time be affirmed that the persistent infections described here operate by the same mechanisms as latent infections by the Herpeto-viridae in vivo, this system would appear to be a useful tool to investigate this important phenomenon.
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The work described in the five papers presented in this dissertation has both immediate practical application and long-term theoretical interest.

The immediate practical application is related to the observation that a sow infected with pseudorabies virus when it was a baby pig, 19 months previously, shed virus in the postparturient period. It is clearly necessary that this single observation should be confirmed by other investigators before it is given too much credence, but if it is confirmed it points to a major difficulty in any pseudorabies eradication program, in that the virus may be carried in clinically recovered breeding stock and may be actively shed under conditions not yet adequately elucidated. In this instance, shedding was postpartum and was detected over a 6-day period. Fortunately, such potentially dangerous stock may very probably still be identified by the presence in their sera of circulating antibodies, and the present policy in the United States of testing for these antibodies in animals intended for sale to other farms as breeding stock would appear to be soundly based.

Also of immediate practical application is the isolation from a herd of pigs in northern Iowa of a relatively nonpathogenic strain of pseudorabies. While naturally any farmer would, if he has to have a disease in his livestock, prefer it to be of a mild and nonpathogenic nature rather than being due to a highly pathogenic strain, it is helpful in any disease eradication program if the disease produces prominent and
characteristic signs so that it may be promptly and unequivocally recognized. The presence of the IA80 strain in Iowa causing a form of the disease which is not easily clinically recognized means that great care has to be taken in diagnosis, and continuing reliance must be placed upon the meaning of seroconversions in herds with no apparent clinical signs. There is certainly no need to postulate for the moment the existence of serological cross-reactions with other microorganisms. The work reported does not, of course, prove that this cannot or does not exist, but it would seem that for the moment that the regulatory authorities may continue to assume that the presence in pig sera of agents which neutralize pseudorabies virus indicates previous infection with that disease agent.

The work reported has several aspects of theoretical interest. The existence of latency with pseudorabies disease and the shedding of infectious virus after provocation is similar to that observed with other of the Herpetoviridae, and reinforces the concept that there is a universality to the phenomenon.

The possible correlation of the recrudescence of the virus with sex hormones or oxytocin is of great interest. Recrudescence of Herpes simplex type 1 virus has been attributed to menstruation and coitus, and one cannot help but wonder if the ecology of the venereal Herpes simplex type 2 is not also influenced by sex hormones. It follows, therefore, that the experiments described here using hormonally simulated pregnancy and parturition in the pig are of some importance. It is unfortunate that the results have so far been inconclusive. It is probable that
latent infection was not established in the pigs, and so obviously the hormones were actually untested as recrudescing agents. This work is presently being repeated. In view of the expense and time involved in experiments using pigs, it would be appropriate to try similar experiments using \textit{Herpes simplex} virus in laboratory animals.

The sow which shed virus in the postparturient period was originally one of a litter of pigs infected when 6 weeks old. Six of 7 of her littermates were subsequently shown to have become latently infected (Beran et al., 1980). The 4 pigs used in the experiment to test the effect of hormonal simulation of pregnancy and parturition were tested with negative results for the presence of virus by culture of tonsillar biopsy samples before this experiment (Davies and Will, Unpublished), and by in vitro organ culture of tissues collected at necropsy at the end of the experiment. Why in one case an attempt to establish latency should have been successful with 7 of 8 pigs, and in the other case it should have been quite unsuccessful with 4 pigs is not known. Clearly, such a knowledge would be central to our understanding of the phenomenon. Although neither of the following factors were applicable in the above experiments, a start has been made on the elucidation of this problem by testing the effect of age at time of infection upon establishing latency, and by comparing whether there is differing ability for infections to become latent between S62/26 and IA80 strains of virus.

Also of theoretical interest was the apparent ability of IA80 virus to cross the placenta of a sow and to thereby infect a fetus in utero, without killing it. This virus, when experimentally injected
intramuscularly into young pigs was shed from the nostrils for up to 14
days later, and caused seroconversion of incontact sentinels. It is in­
teresting to speculate that there might be vertical transmission from
dams to their young pigs and that the virus might then be shed from them
to infect other pigs. Even more interesting is the possibility that such
virus, acquired in utero, might enter into a latent form and might only
be shed as infectious virus at much later dates, perhaps even infecting
in later gestations. Such speculation is quite unproven, but would be
of tremendous interest to investigators of all the Herpetoviridae. Work
is presently in progress to test the vertical transmission of IA80 strain
from pregnant sows to their offspring. It would also be interesting to
test for vertical transmission of Herpes simplex in say venereally in­
fected mice.

The investigations aimed at establishing persistent infections in
vitro were successful, but like the numerous experiments performed by
other investigators, their relationship with latency in host animals was
unclear. Further investigation of in vitro systems appears proper, be­
cause final understanding of any mechanisms of latency at cellular level
is likely to depend upon finding, in cell culture, the conditions for
rendering cells permissive or nonpermissive at will, and defining the
host factors, operative and inoperative, in each instance (adapted from
Roizman, 1974). Work reported here differed from that of most previous
investigators in that it used cells taken directly from the living animal,
rather than continuous cell lines, and so at the start of the experiment
at least cellular transformation had not taken place. In vivo,
pseudorabies virus has a different effect upon the porcine organism as compared to other mammalian species. Whether this difference may be carried over to the in vitro system is presently under investigation.

The Herpetoviridae are a most significant group of viruses, and amongst them, pseudorabies is of great importance. It is hoped that the investigations reported in this dissertation will help all those who are working to treat, control and eradicate the diseases caused by them. Studies on the mechanisms of latency in all the Herpetoviridae are still in very early stages. It is hoped that findings reported here on pseudorabies virus will have application to greater understanding of the Herpetoviridae and will help to stimulate further studies on pseudorabies virus as a member of this viral family.
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Some two years ago, when I completed my M.S. thesis, I was happy to acknowledge the cheerful technical help of Mrs. Dorothy (Dee) Murphy, and I commented that she kept smiling in the face of all the disasters which overtook us in the laboratory. I am pleased to say that she has continued to work as my colleague during our continuing research program. For some reason we do not appear to have had so many disasters to contend with while we have been doing our Ph.D. as when we did our M.S. Perhaps this is an illusion, and we are just hardened to them and so do not notice them. Another possibility is that some of the difficulties have been smoothed away by the efficient technical help of Mr. Charles Gold and Miss Virginia Smith. I thank all three of them.

My writing is notoriously illegible and may readily be mistaken for
a cipher. I thank my typists, Mrs. Heidi Anderson, and Mrs. Gwen Ethington for having so patiently and efficiently converted it into fine clear print.

I shall be leaving Ames within several weeks of writing these acknowledgments. Naturally I shall be happy to go home. My happiness, however, will be tempered by a sadness arising from the fact that I shall be leaving so many friends in Ames. I thank them all and wish them well.