A study of the survival of Aujeszky's Disease (pseudorabies) virus outside the living host

ISU 1979 D287 c.3

by

Edward Bryan Davies

A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Veterinary Microbiology
and Preventive Medicine
Major: Veterinary Preventive Medicine

Signatures have been redacted for privacy

Iowa State University
Ames, Iowa

1979

1251473

TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF THE LITERATURE	2
Species of Animals Affected	2
Symptoms and Course of Disease	3
History of the Disease in Iowa	6
Mechanisms of the Spread of the Disease in Pigs	12
Survival of the Virus Outside the Living Host	18
MATERIALS AND METHODS	24
Media	24
Cell Culture	26
Virus	27
Sterilization Processes and Cleansing of Glassware	28
Viral Assay by Plaque Counting Technique	28
Adjustment of pH of Suspensions Containing Virus	30
Temperature Adjustment	31
Drying Virus	33
Ultraviolet Light	34
RESULTS	40
The Effect of Constant Temperature and Various pH Levels Upon the Survival of Virus	40
The Effect of Temperature Fluctuation and pH Level Upon the Survival of Virus	59
Storage of Virus Suspensions by Freezing	59
The Effect of Drying and pH Level Upon the Survival of Virus	74

Page

The Effect of Ultraviolet Light and pH Level Upon the Survival of Virus 74

DISCUSSION 81

Estimation of the Titer of Virus in a Suspension 81

Certain Aspects of Experimental Design 82

Survival of Virus at Constant Temperatures Above the Freezing Point 84

The Effect of Fluctuating Temperature Upon the Survival of Virus 87

Long-Term Storage of the Virus in the Laboratory 90

The Effect of Drying Upon the Virus 91

The Effect of Ultraviolet Light Upon the Virus 93

Limitations of This Work 94

SUMMARY 96

BIBLIOGRAPHY 97

ACKNOWLEDGMENTS 103

LIST OF TABLES

Page

Table 1. Preparation of Minimum Essential Medium (MEM). 36

Table 2. Preparation of Saline G. 37

Table 3. Preparation of trypsin-versene solution. 38

Table 4. Preparation of glassware cleansing solution. 39

LIST OF FIGURES

Page

Figure 1. The effect of a constant temperature of 37°C upon the titer of suspensions of Aujeszky's Disease virus held at various pH levels. 41

Figure 2. The rate of loss of titer of Aujeszky's Disease virus suspensions at various pH levels held at a constant temperature of 37°C 43

Figure 3. The effect of a constant temperature of 25°C upon the titer of suspensions of Aujeszky's Disease virus held at various pH levels. 45

Figure 4. The rate of loss of titer of Aujeszky's Disease virus suspensions at various pH levels held at a constant temperature of 25°C 47

Figure 5. The effect of a constant temperature of 17°C upon the titer of suspensions of Aujeszky's Disease virus held at various pH levels. 49

Figure 6. The rate of loss of titer of Aujeszky's Disease virus suspensions at various pH levels held at a constant temperature of 17°C 51

Figure 7. The effect of a constant temperature of 4°C upon the titer of suspensions of Aujeszky's Disease virus held at various pH levels. 53

Figure 8. The rate of loss of titer of Aujeszky's Disease virus suspensions at various pH levels held at a constant temperature of 4°C. 55

Figure 9. The minimum rate of loss of titer of Aujeszky's Disease virus suspensions held at optimal levels of pH at different constant temperatures. 57

Figure 10. The effect of ten temperature fluctuations between 6°C and 37°C, compared with the effect of constant temperatures of 6°C and 37°C, upon the titer of Aujeszky's Disease virus suspensions held at various pH levels. 60

Figure 11. The effect of freezing and thawing once, twice and three times, compared with the effect of not freezing, upon the titer of Aujeszky's Disease virus suspensions held at various pH levels 62

Page

Figure 12. The effect of three temperature fluctuations between -90°C and -13°C, compared with the effect of a constant temperature of -90°C, upon the titer of Aujeszky's Disease virus suspensions held at various pH levels. 64

Figure 13. The effect of storage at -90°C, compared with the effect of storage at -13°C, upon the titer of Aujeszky's Disease virus suspensions at various pH levels. 66

Figure 14. The effect of a single rapid freezing to -90°C, compared with the effect of maintenance at 4°C, upon the titer of Aujeszky's Disease virus suspensions at various pH levels. 68

Figure 15. The difference between titers of Aujeszky's Disease virus suspensions rapidly frozen once to -90°C and held at a constant temperature of 4°C, at various pH levels. 70

Figure 16. The effect of storage at -90°C for a period up to 29 days upon the titer of Aujeszky's Disease virus suspension at various pH levels. 72

Figure 17. The effect of drying at temperatures of 14°C, 22°C, and 37°C, upon the titer of Aujeszky's Disease virus suspensions supported on glass, at various pH levels. 75

Figure 18. The effect of drying at a temperature of 37°C upon the titer of Aujeszky's Disease virus suspensions supported on gelatin, at various pH levels 77

Figure 19. The effect of exposure to ultraviolet light for periods of 20, 40 and 60 minutes upon the titer of Aujeszky's Disease virus suspensions held at various pH levels, compared with the effect upon the titer of control suspensions 79

INTRODUCTION

In 1902, Aujeszky, working in Hungary, investigated a disease of domestic animals which was characterized by nervous symptoms. Using material derived from a bovine animal, a dog and a cat he was able to transmit the disease by the subcutaneous route to rabbits, dogs and guinea pigs. Most importantly, he was able to show by its shorter incubation period, intense local pruritis, relatively short course and absence of paralysis that the disease was different from rabies. This allowed future workers to study the disease as an entity in its own right, rather than as previously, as a confusing variant of rabies.

In recognition of his pioneering work the disease which he described is now known in Europe as Aujeszky's Disease by speakers of all the European languages. In North America, the name pseudorabies is usually used in scientific circles, and in nonscientific circles, the name "Mad Itch," dating from the last century, still retains its popularity. The name Infectious Bulbar Paralysis, used by Marek, 1904, as cited by Baskerville et al. (1973), is now out of vogue.

REVIEW OF THE LITERATURE

Species of Animals Affected

Over the following years it was noted that besides occurring naturally in cattle, dogs and cats, as described by Aujeszky, the disease also occurred naturally in pigs (Von Ratz, 1914) and sheep (Szilard, 1927), both these authors cited by Galloway (1938). Of the common domestic animals, the horse uniquely appears to be somewhat resistant to the disease, even when experimentally inoculated. In one experimental study, infection was not produced in any of 6 horses or 3 mules tested (Braga and Faria, 1934). The resistance in horses is not however, complete. Schmiedhoffer's (1910) horses were less fortunate. Three out of 6 died.

Knowledge of the susceptibility of wild animals to the disease is of great epidemiological interest, and several extensive series of experiments have been conducted by a number of workers on a wide variety of mammals, birds, and other animals, to ascertain whether they could contract the disease.

These experiments, and reports of natural cases, show that the mouse, the Norwegian rat, the hedgehog and the jackal (Remlinger and Bailly, 1934), the marmoset monkey, the porcupine and the opossum (Braga and Faria, 1934), the roe deer (Nikolitsch, 1954), the mink, arctic fox and silver fox (Ljubashenko, et al., 1958), and the rhesus monkey (Karasszon, 1965) can all be infected with the disease. The susceptibility of North American wild animals was studied by Trainer and Karstad (1963), and they were able to infect skunk, muskrat, raccoon, badger, woodchuck, white--

tailed deer, cotton-tailed rabbit and red fox.

Not all mammals however are susceptible. Nicolau et al. (1937) failed to infect chimpanzees, and human beings appear to be refractory. The description by Tuncman (1938) of the disease in people has not been generally accepted.

Besides mammals, certain birds and fowl such as the pigeon, goose, mallard duck, buzzard, sparrow hawk and chicken (Remlinger and Bailly, 1934) and the turkey (Remlinger and Bailly, 1939) can be experimentally infected. Schneider and Haslett (1978) however failed to infect the European starling.

Attempts to infect other forms of animal life such as the tortoise, toad and frog (Remlinger and Bailly, 1934), and the snake (Braga and Faria, 1934) have been unsuccessful.

Symptoms and Course of Disease

These topics have been reviewed by a number of workers over the years and these notes draw especially upon the review by Galloway (1938) for the views of earlier workers in this field and upon the reviews by Baskerville et al. (1973) and Gustafson (1975) for later views.

The symptoms and course of the disease in the pig differ from those observed in other animals in that pruritis is not a prominent feature. It is generally observed that the disease affects young pigs of less than four weeks of age more severely than it does older fattening pigs. Except in gestating female pigs, two clinical forms predominate, a rhinitis with or without pneumonia or else a generally more severe form with nervous

symptoms including incoordination and convulsions. Both forms may be accompanied by severe pyrexia, and both may lead to death. Vomiting is not common, but it has been recorded (Burggraaf and Lourens, 1932, as cited by Baskerville et al. (1973); Kretzschmar and Schulz, 1964), and its significance will be discussed later.

In the past it was considered that fattening pigs would be little affected by the disease, but in recent years pigs of this age have been more severely affected, sometimes with high mortality, presumably due to the prevalence of more pathogenic strains of virus.

Abortion and the production of mummified fetuses were first reported in sows affected with Aujeszky's Disease in Ireland by Gordon and Luke in 1955. This work was confirmed by Csontos et al. (1962) in Hungary and has been generally accepted more recently by a number of workers, including Kluge and Maré (1974) and Maré et al. (1976) in the United States. The appearance of the disease in breeding herds is now recognized to be a serious problem. During an epidemic, if the sow does not abort or produce mummified fetuses at full term, the young pigs are likely to succumb to the disease in their early weeks of life.

Unlike pigs, cattle are almost invariably very seriously affected if they contract the infection; intense pruritis accompanied by self-mutilation is a promiment symptom in this species. The animal may lick a portion of its skin to such an extent as to denude it of hair or it may rub itself on a fence or gate post in such a frenzy that it completely abrades the surface of the skin. The name "Mad Itch" is a very fair description of the disease in cattle. It is a name that is never used

when talking about Aujeszky's Disease in pigs. In cattle, death is the usual outcome, occurring in most instances within 48 hours of first appearance of symptoms.

Sheep, like cattle, may be severely affected by this disease and pruritis is again a prominent symptom. Marcis, 1933, as cited by Galloway (1938), recorded a natural outbreak of the disease in these animals in which they gnawed at their hind legs, scratched their bodies with their feet and bit and pulled out wool from other parts of their bodies with their teeth. The course of the disease was rapid and animals died within 8-24 hours of showing symptoms. More recently Ivanov et al. (1968) investigated 4 epidemics in flocks of sheep. Of 1,974 animals, 488 contracted the disease and died.

In dogs the disease causes depression and is associated with pruritis in many cases. It is frequently fatal. Galloway (1938) noted that in naturally occurring cases of the disease in dogs, and in cases induced by feeding infected material, the animals rubbed or bit their lips until the blood flowed. This observation was also noted by Cassells and Lamont (1942), who also commented on the rapid course of the disease in dogs, death in their cases occurring within 24 hours of initial signs, and being accompanied by great thirst. Infection in dogs may very readily be associated with the consumption of carcasses of animals which have died of Aujeszky's Disease. Gore et al. (1977) reported a case in England in which 11 of 51 Harrier hounds fed carcasses from a pig fattening unit died. It is interesting to note that until the trace-back following the recognition of the disease in the dogs, the disease had

not been noticed in the pig herd.

In cats, the disease follows a similar course to that in dogs, excepting that it is usually more severe and more rapidly fatal.

History of the Disease in Iowa

In the Cultivator (Albany) of 1839 there appeared a report of an outbreak of what was undoubtedly Aujeszky's Disease even though of course it was not known by that name. The outbreak occurred in Washington County in Iowa, and the report stated "there is a complaint raging among the cattle of this county called the Mad Itch, which appears to be incurable. . . . It takes the cattle with an itching on the nose or around the horns, they then commence rubbing and throwing their heads and frothing at the mouth, and in about 24 hours they are dead."

Ninety-two years later, Shope (1931) by interesting chance investigated a severe outbreak of Mad Itch in cattle also in Iowa, and was able for the first time to identify the disease as being that described by Aujeszky. Shope quite enthused about the episode, and stated "the disease is so rare as to constitute a veterinary curiosity."

It is interesting to speculate as to whether the disease remained endemic in Iowa for the 92 years separating these two dramatic documented outbreaks. Quite possibly it did, for while dramatic herd outbreaks of the pruritic syndrome in cattle were probably rare, sporadic cases of "Mad Itch" were not unknown. If so, where was the disease during the period between the conspicuous cases in cattle? Presumably it survived in an unrecognized endemic form in the pig population.

As has been discussed, pruritis is not a prominent symptom of Aujeszky's Disease in pigs, and so it is not easily identifiable as being the same as a concurrent disease in cattle. Was there any illness during this period which could have been this disease in an unrecognized form?

The answer is almost certainly in the affirmative, for the pig population in America during the latter part of the 19th century had more than its fair share of ailments, and a disease such as Aujeszky's Disease could well have passed unrecognized and almost unnoticed. This view was expressed by the Commissioner of Agriculture in his report on animal diseases of 1866. He stated "Of all domestic animals, those (diseases) of this genus are evidently less thoroughly understood than those of any other farm stock. Ideas on the subject are in a singular state of confusion, and remedies are countless in number and most incongruous in character. If the symptoms were actually noted, it would probably be found that several kinds of 'hog cholera' - as every prevalent disease of the hog appears to be called - are uniting in the mischief produced" (abstracted by Brierer, 1939).

In the same report, the commissioner noted that 15% of the pigs in Jefferson County, Iowa, died during that year. In the face of such mortality, it is quite easy to imagine a disease with no characteristic symptoms passing unrecognized as a separate entity.

The association of "Mad Itch" in cattle with the presence of pigs may have been noted but was not properly understood early in this century. Nelson, 1910 (excerpt in Brierer, 1939), discussing the etiology of the

disease stated it was due to "consuming corn husks, corn stalks, corn cobs that have been fed to hogs and partially masticated and dropped out of the mouth or swollen (swallowed) and afterwards voluntarily ejected from the stomach. . . ." It can only be speculated that he may have seen pigs contaminating cattle fodder and not have understood the significance of the occurrence. Alternatively, he may only have observed a coincidental event.

As has already been mentioned, Shope investigated in 1931 what he then referred to as an outbreak of "Mad Itch" in dairy cattle in Johnson County, Iowa. In this and a subsequent series of papers (Shope, 1931, 1932, 1934, 1935a and 1935b) he was able to show that the disease was the same as the one described in Europe by Aujeszky, that it occurred in a relatively mild and not easily recognized form in pigs, that it was transmitted from pigs to cattle, and that in cattle it developed into the intensely pruritic and fatal form which had been known to farmers and veterinarians for decades. In his paper of 1932 he commented "in any future publications the "Mad Itch" virus will be designated as Pseudorabies Virus (Iowa Strain)." He may therefore be thanked for the removal of the journalistic "Mad Itch" from the scientific literature. It is a pity however that he chose to replace it with the equally dramatic and scientifically unmeaningful "pseudorabies," rather than honoring Aladár Aujeszky.

The position in the United States in the 1930s was that Aujeszky's Disease in pigs was regarded as being mild and unimportant. In the United Kingdom, the same view was held. Galloway, writing in 1938,

commented that when he started working on the disease in 1930, "it was not generally known that there was an entity, Aujeszky's disease," and he could quite reasonably remark that "a review of the literature does not lead one to share the pessimistic view of Köves (1935)." This pessimistic view, out of tune with the best opinions in the English-speaking world, stated "we believe that in future we shall have increasing records of this disease and shortly it will be proved that we are faced with one of the diseases most widespread throughout the world, a disease which we have known for a long time, but under another name" (Köves, 1935, as cited by Galloway (1938).

It is probable that Köves fairly represented the Central European view of the time. Certainly workers in that geographical area appeared to take the disease more seriously than did those in the United States and the United Kingdom. The reason for this is not certain. It may be that they had a more virulent, more pathogenic strain of the virus to contend with in Central Europe. Comparisons which may be made now between strains of Central European and American or British origin would tell us little of the position forty years ago, because all the early strains may have changed in this period. On the other hand the extra interest shown by the Central European workers in Aujeszky's Disease may have been simply because they had at that time more available and better-trained veterinary manpower and were under less pressure to investigate other diseases. There are many possible reasons why they may have exhibited more interest than workers in America and the United Kingdom. The greater concern which grew out of their studies may have been

warranted, or it may have been whim.

In the United States and the United Kingdom the view that Aujeszky's Disease was not serious in adult pigs, as expressed by Galloway and Shope, was accepted without question for 20 or 30 years. Then a number of papers began to be published which spoke of higher than usual pathogenicity and death in older pigs.

In 1963, in Indiana, there was a small epidemic of Aujeszky's Disease affecting three herds of pigs. The epidemic was unusual in that mortality was as high as sixty percent (Saunders et al., 1963). A later report reviewed the cases noted in this same State during the period January 1962 - October 1964 and again concluded that there was a higher than usual pathogenicity (Saunders and Gustafson, 1964). The subject has been reviewed by Stewart (1975).

Over the succeeding decade, interest in Aujeszky's Disease grew in the United States, particularly in the major pig-producing states of the midwest, and reports of the disease increased dramatically. Stewart (1975), reporting on the widespread nature of the disease, noted that during the period from fiscal year 1966 through fiscal year 1972, the National Veterinary Services Laboratory, Animal and Plant Health Inspection Service (NVSL, APHIS), U.S. Department of Agriculture, had diagnosed Aujeszky's Disease in pigs in eleven states.

In Iowa, Kluge and Maré (1974) reported on a strain of Aujeszky's Disease virus which was able to kill five of twelve pregnant gilts, a death rate in adult pigs which at one time would have been considered impossible, and Hill and Maré (1975) concluded that both the incidence

and the severity of the disease were increasing in the United States. Dillman and Andrews (1975) agreed with this conclusion. They noted that the rate of laboratory diagnosis in Iowa increased sixfold in the first 9 months of 1974 as compared with the average rate of the previous four years, and that the pathogenicity also increased. Beran et al. (1976) documented the substantial financial losses associated with the disease in the midwestern United States.

Aujeszky's Disease became a popular topic of conversation. Meetings attracting speakers from abroad as well as from the United States were held to discuss the problem (Symposium on Pseudorabies [Aujeszky's Disease], 1975 and Pseudorabies Fact-Finding Conference, 1977), and numerous speakers and groups expressed their concern and interest in the problem.

This growing concern and interest were not confined to the United States. Baskerville et al. (1973) noted a surprisingly high mortality in pigs 14-20 weeks of age in the United Kingdom, and workers in that country also had to accept that Aujeszky's Disease could have serious consequences in pig herds.

And so, forty years after he expressed them, the "pessimistic views" of Köves, 1935, (as cited by Galloway, 1938), that "we believe that in future we shall have increasing records of this disease" have come true.

In concluding this section on the history of the disease in Iowa, it is proper that, to give a properly balanced view, a note of caution should be expressed. It is dangerous to be dogmatic when discussing reports of disease incidence and severity. The number of reported cases

in Iowa has definitely increased. That is a matter of public record and cannot be disputed. The increased number of recorded cases may however in part be due to a greater awareness of the disease and to more thorough reporting. Now that hog cholera has been eradicated there is, so to speak, a vacuum in dramatic pig diseases, and records of Aujeszky's Disease may have obligingly expanded to fill the void.

When speaking of the severity of the disease greater certainty can be expressed. Unless there was a great failure in diagnosis in investigations of abortions and deaths in adult pigs and of massive fatal epidemics in newborn pigs twenty years ago, there has been a significant increase in pathogenicity of the disease in recent years.

Two points may be safely stated dogmatically. Aujeszky's Disease is presently attracting great interest in Iowa, and pig producers want something done about it.

Mechanisms of the Spread of the Disease in Pigs

Aujeszky's Disease is an infectious disease caused by a virus of the Herpetovirus group. Taxonomically the virus is classified as a possible species, porcine herpesvirus 1 in the genus _Herpesvirus_, family _Herpetoviridae_. The virion comprises a DNA core inside an icosahedral capsid, the whole being enclosed in an envelope (Kaplan, 1969). The overall diameter of a virion is approximately 150-170 nm (Fenner et al., 1974).

The disease cannot occur in the absence of the virus and while, as with many other diseases, there are many predisposing factors, to the best

of present knowledge the virus is able to produce the disease without the presence of any other etiological agent.

A herd of infected pigs may be a major source of infection, both to other pigs and to other species of animals. The mechanism whereby the disease spreads in the first instance to the susceptible pigs is therefore of great interest.

During the course of the disease in pigs, rhinitis and pneumonia are usually prominent symptoms, and virus may be found in the nasal and oral discharges. Infection may be readily induced in susceptible pigs by the instillation of virus-containing material into the nostrils. It is reasonable to believe that in nature infection occurs as a result of the close nasal and oral contact practiced by pigs. Nikitin (1961) claimed that recovered pigs could excrete virus in their urine for periods of up to 186 days. Other workers, however, have not been able to repeat this work. McFerran and Dow (1965) did not find detectable virus in either urine or feces and Beran et al.[1] did not find it in kidney or intestinal wall. Fecal excretion is unlikely on theoretical grounds (Fenner and White, 1976). They point out that enveloped viruses are likely to be destroyed by the bile salts which will be present in the alimentary canal. Infected sows have been shown to secrete virus in their milk and pigs suckling them contracted the disease (Kojnok, 1957). It does seem, however, that the main excretion of the virus from infected pigs is in the nasal and oral secretions, and that urine, feces and milk

---

[1]Beran, G. W., P. V. Arambulo III, E. B. Davies, and L. A. Will, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa. Publication Pending.

play at the most only a minor part in the dissemination of the disease.

Just because pigs may readily infect each other by direct contact does not immediately make apparent the sources of virus in outbreaks or prove that direct contact is the only means whereby infection may spread. In a study of over 600 outbreaks of Aujeszky's Disease in Iowa in 1976 through 1978, Owen and Beran[1] noted that in only about 25% of the cases was it possible to correlate the outbreak of the disease on a hitherto uninfected premise with the introduction of pigs. In 75% of the outbreaks the route of introduction of the virus was not explained. The identification of sources of infection is of prime importance if any success is to be attained in controlling the spread of the disease.

There are a number of possible ways in which virus may be introduced into a healthy herd of pigs without it being carried by clinically or inapparently infected pigs.

One way is that wild life may transmit the virus from one pig herd to another. McCrocklin (1976) and Kanitz (1977) believe that this is a possibility, particularly with the raccoon, which has the well-known habit of invading pig pens to feed on their food. Such an animal could feed with infected pigs, become infected and live long enough to travel to another farm to shed virus, die, and perhaps be consumed by previously healthy pigs. Domestic dogs and cats, and the feral dogs and cats found on many farms may also possibly spread the disease in this way.

A second possible way in which the virus may be transmitted in the

[1] Owen, W. J., and G. W. Beran, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa. Publication Pending.

absence of the infected pig is by arthropod vectors. Mašić and Petrović (1961) tried to infect mice by intracerebral and rabbits by intramuscular injections of a suspension prepared from lice (Haematopinus suis) which had been fed on infected pigs, but with no success. Glukhov (1971) however, fed ticks (Dermacentor marginatus) on infected rabbits and showed that they were able to harbor virus for up to 60 days, and he was able through them to transmit the disease to young pigs. Despite this work however, Aujeszky's Disease is not considered to be transmitted by biting arthropods to a significant degree. The fact that the disease is able to spread in parts of the world and in climatic conditions and seasons not favorable to flying arthropods is evidence against their great involvement, and arthropods which tend to remain in close association with the pig, such as lice, can hardly be of greater importance in the spread of the disease than the pig itself.

A third possible way in which the disease may appear in pig herds without the recent introduction of clinically infected or incubating pigs is by a recrudescence of a hitherto unnoticed latent infection. It is well-known that certain members of the Herpetovirus group are able to lie dormant in the tissue of an apparently healthy host, only to become reactivated under conditions not fully understood, but often associated with stress. Human Herpesvirus 1 (Herpes simplex), the virus causing the common "cold sore" or "fever blister" in people is a well-known example. If Aujeszky's Disease virus has similar properties it could explain the appearance of the disease in pig herds which have had no recent contact with other pigs.

To test the persistance of infection, McFerran and Dow (1964) stressed previously infected and fully-recovered pigs by artificially induced anaphylaxis and diet changes, and Sabó (1969) stressed them with a combination of cortisone treatment and temperature and diet changes. None of these workers was able to demonstrate the shedding of virus.

Howarth however, while studying the naturally-occurring disease in outbreaks in garbage-fed pigs in California in 1969 was convinced that apparently healthy pigs could shed virus and so infect other pigs and he concluded from his studies that climatic stress was an important factor in the initiation of the virus release.

Beran et al. (1976), studying the disease in a field epidemic in Iowa, obtained evidence that a carrier state could exist for at least 4 months, with subsequent shedding of virus and infection of susceptible pigs, particularly under the stress of movement, farrowing or lactation.

The work of these authors is supported by that of Simeonov (1973). He was able to recover virus from the tonsils of pigs infected up to 2 months and sometimes up to 4 months previously.

While experimental work has not yet succeeded in eliciting the release of virus from the living carrier pig, it has succeeded in eliciting the release of virus from isolated fragments of pig tissue maintained *in vitro* in nutrient medium (Sabó and Rajčáni, 1976 and Beran et al.[1]), so there is no doubt that the virus does persist in the tissues of infected

---

[1]Beran, G. W., P. V. Arambulo III, E. B. Davies, and L. A. Will, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa. Publication Pending.

pigs.

To summarize the position, Aujeszky's Disease virus has been shown by virological techniques to persist in the tissues of apparently healthy pigs long after their recovery from clinical disease. There is extensive epidemiological evidence that pigs may on occasion shed this virus and so infect other nonimmune pigs. There is reason to believe that this happens particularly with sows at the time of parturition, but recrudescence of active shedding has not been proven by viral isolation from field pigs or been induced artificially from live pigs in the laboratory.

Finally, a fourth possible way in which infection may be transmitted in the absence of the infected pig is that virus which has been excreted from a diseased pig may survive in the environment and be transferred on inanimate fomites - automobile wheels, dirty boots, dust, bedding and such like - to healthy susceptible pigs. This means of spread is fundamentally little different from direct pig-to-pig spread. After all, even in the direct contact of one pig with another, the virus must at some point in time be outside the tissue of the pigs and so must be able to exist in the environment for some period of time at least. Clearly it would be to the advantage of the virus to be able to survive for greater rather than shorter periods and to be able to survive on a variety of inanimate objects so that if immediate contact is not made with a susceptible host, the virus may survive to infect at a later date. The ability of the virus to survive outside the host is of fundamental importance in epidemiological studies and the study of this ability forms the

major part of this dissertation.

Survival of the Virus Outside the Living Host

In the previous section, the work of Owen and Beran[1] in which in a large study in Iowa they were not able in 75% of the outbreaks to associate the disease with the introduction of possibly clinically affected animals was noted. Other possible means whereby virus may be introduced onto a premises, namely viral contamination on fomites of some sort or infection in wild life, in arthropods and in latently affected pigs have been suggested.

This section will be concerned only with the ability of the virus to survive outside a living host, and as a starting point, will consider viruses in general, rather than Aujeszky's Disease virus in particular. Fenner and White (1976), in a review of viruses of medical interest, stated that such viruses are notoriously heat-labile, particularly those which are enveloped. At high temperatures viruses are quickly inactivated by denaturation of the capsid proteins. Even at the body temperature of their host, to which one would expect pathogenic organisms to be well-adapted, they lose infectivity, but in a living host this loss is likely to be more than adequately compensated by multiplication. As a rule of thumb, Fenner and White suggested that the half-life of viruses at 60°C is measured in seconds, at 37°C in minutes, at 20°C in hours and at 4°C in days. A half-life of hours may be perfectly adequate to insure

[1] Owen, W. J., and G. W. Beran, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa. Publication Pending.

adequate survival during transmission for viruses in secretions or fluids excreted from the host at concentrations well in excess of 1,000,000 infectious units per ml. It should however, be noted that there are exceptions to these generalizations. Certain of the slow viral encephalopathies such as scrapie in sheep and goats and kuru and Creutzfeldt-Jakob disease in people are caused by agents which are highly resistant to heat (Fenner et al., 1974).

Again speaking generally, Fenner and White stated that viruses prefer a physiological pH but that their limits of tolerance are fairly wide and that the enveloped viruses are susceptible to lipid solvents and so do not survive in the alimentary tract.

Turning to studies specifically relating to the survival of Aujeszky's Disease virus, we have a number from which to choose. Most have been conducted in Central Europe and they appear to be mainly of a practical field-application type, rather than being basic studies under controlled conditions.

Solomkin and Tutushin (1956) studied the survival of the virus under various climatic conditions on a variety of fomites such as hay, straw, sacks, twine, wooden boards, potatoes, grain and oats. They stated that in winter (average temperature -8°C) the virus survived 35-46 days; in summer (average temperature 25°C) 10-30 days; and in autumn (average temperature 14-18°C) 5-30 days.

Ustenko (1957) noted that the virus survived on wooden boards for 24-28 days in summer and 46-49 days in winter and went on to recommend that thorough disinfection should be practiced with this disease.

In a subsequent paper, the same author (Ustenko, 1958) again examined

the survival of virus on wooden boards and this time noted that it lived 14-37 days indoors, the period depending upon the temperature and humidity, and 20-38 days out-of-doors. Survival decreased in the presence of dung. In the same paper the author also noted that the virus would survive 24-28 days in rabbit skin pickled in brine, which may serve to warn that it may live quite well in rather unexpected places.

Atanosova (1972) was concerned about the survival of virus in the urine of pigs and sheep. Doubt has already been expressed that viable virus is excreted in any sizable amount in the urine of pigs so this would not appear to be a major concern, excepting in so far as the virus in nasal and oral secretions may contaminate urine collected in a pig pen. If it does, according to this author, it may survive at least 53 days at 4°C and 18 days at room temperature. In sheep's urine however inactivation occurred within 3 days.

In contrast to these field-oriented studies, Žuffa and Škoda (1962) investigated the survival of the virus under more controlled conditions. They noted that it was stable at pH 5.0-9.0 at a temperature of 4°C and that when frozen it was also stable at a temperature of -60°C, but that it was not stable at a temperature only a little below freezing, -15°C, nor was it able to withstand repeated freezing and thawing. Maré (1975) agreed that the virus was not stable at a temperature only a little below freezing. According to him it may be inactivated at 0°F (-18°C) in a matter of weeks.

Besides the studies described above, in which the ability of the virus to survive under fairly natural circumstances has been investigated,

in the last several years two groups of workers have studied the effect of disinfectants. In Russia, Polyakov and Andryunin (1974) noted that Aujeszky's Disease virus would live five days in slurry (liquid manure) in summer in a warm climate, but that it could be destroyed by a variety of disinfectants or by changing the pH to 5.0. In Italy, Venturoli (1975) was able to destroy the virus in 5 minutes using a 10% calcium hydroxide solution or a 0.25% solution of a mixture based upon formaldehyde and a quaternary ammonium salt.

Up to now survival of the virus outside the living host has been considered only outside animal tissues. If however an infected animal dies, or is killed, some virus will then find itself inside animal tissue but it will not be able to replicate in that tissue, because the essential chemical processes of the host on which it depends will have become disrupted. Essentially therefore on the death of an animal its tissue becomes just like any other external environment and the virus in it must survive as best it can, without replenishment. Field evidences have demonstrated that virus may remain viable in the carcasses of dead pigs because these have been shown able after a period of time to infect a pack of hounds (Gore et al., 1977). Ustenko (1958) investigated this and noted that the virus would live in the muscle of carcasses for 11-18 days at summer temperatures and from 21-36 at spring temperatures. Akkermans (1974) was also concerned about this problem in relation to human health and the consumption of carcasses of cattle affected with Aujeszky's Disease. He recommended removal of the brain and spinal cord and the sale of the meat in small portions.

While the majority of researchers have been concerned in knowing how long the virus might survive so that they may use the information to break a train of transmission, some workers have been interested in knowing how long the virus may survive under particular conditions because they hope to be able to use this information to identify a particular strain of virus and distinguish it from another differently surviving strain. Such an ability would be of great value in epidemiological tracing. This aspect of viral survival has been studied using Aujeszky's Disease virus by Golais and Sabó (1975), Platt and Maré (1976), and Platt (1977). One of the most important points to come out of their work was that various strains of virus did vary in their ability to survive and in their resistance to temperature.

Other workers have been interested in the ability of virus to survive when subjected to freeze-drying during vaccine manufacture. Scott and Woodside (1976), using one strain of Aujeszky's Disease virus and a variety of suspending media, noted that certain of the media had a significant effect upon the survival of the virus. While admitting that this work refers to a specialized laboratory procedure, it does remind us that the virus is not alone in the environment and may be protected or destroyed by a multitude of chemicals about which we know little or nothing.

In conclusion, we may say that while many workers have investigated the survival of Aujeszky's Disease virus both in the field and in the laboratory, much more work needs to be done if we are to be able to accurately predict whether or not it will survive in a particular circumstance.

In response to this need, workers in this laboratory are tackling the problem in two studies. One, Beran et al.[1] is concerned with the survival of the virus under field conditions. The other, the present study, is concerned with the survival of the virus under laboratory conditions.

It is hoped that, taken together, these studies will be of value to those persons concerned in maintaining the health of our livestock.

---

[1]Beran, G. W., P. V. Arambulo III, E. B. Davies, and P. M. Pitcher, Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa. Unpublished data.

MATERIALS AND METHODS

A major part of the work to be described in this report has consisted in the estimation of the amount of Aujeszky's Disease virus still surviving after various procedures. These quantitative estimations have been carried out in cell cultures using plaque-assay techniques. This section begins with a description of the materials used in the preparation and use of the cell cultures.

Media

Minimum Essential Medium (MEM) was prepared by dissolving proprietary dry packs containing the appropriate salts in triple distilled water and adding sodium bicarbonate. See Table 1 at the end of this section for the full formula and for the name of the supplier of chemicals. The solution was sterilized by filtering through a methyl cellulose membrane filter[1] of pore diameter 200 nm and stored at $4^{o}C$. Small aliquots from each lot were tested for bacteriological sterility by inoculation into blood agar plates. The MEM was made in both single and double strength.

Growth Medium (GM) was prepared from single strength MEM by adding 10% fetal bovine serum[2] and Maintenance Medium (MM) by adding 2% fetal bovine serum. Antibiotics were also added for use in terminal passages of cell cultures which were to be used for virus assay and then discarded,

[1] Millipore Corporation, Bedford, Massachusetts.

[2] Sterile Systems Inc., Logan, Utah.

but not for routine maintenance of cell lines. When used, they were penicillin G,[1] streptomycin,[2] amphotericin B,[3] and gentamycin[4] at levels of 100 units, 100 micrograms, 3 micrograms, and 5 mg for 100 ml of final solution respectively.

Saline G was used as the standard diluent for the storage and dilution of virus. See Table 2 at the end of this section for the full formula and for the name of the supplier of chemicals. The solution was sterilized by filtering through a methyl cellulose membrane filter of pore diameter 200 nm and antibiotics were routinely added at the standard concentrations described earlier for GM and MM. The pH of the saline G was adjusted using 0.1N hydrochloric acid or 0.1N sodium hydroxide solution to pH 7.0 before routine use or to whatever pH was required by the dictates of the particular experiment.

Gum Tragacanth Solution was prepared by slowly adding 1.6 grams of proprietary gum tragacanth[5] to 100 ml of agitated, warm, triple distilled water and was sterilized by autoclaving at a pressure of 20 lbs. per square inch for 30 minutes.

Overlay Medium was used to maintain cell culture monolayers after infection with virus containing solution and to, at the same time, stop the diffusion of virus from an infected to a noninfected part of the

---

[1] Penicillin G Potassium, E. R. Squibb & Sons, Princeton, New Jersey.

[2] Streptomycin Sulfate, Pfizer Laboratories Division, New York.

[3] Amphotericin B (Fungizone), E. R. Squibb & Sons, Princeton, New Jersey.

[4] Gentamycin Sulfate, Schering Corporation, Kenilworth, New Jersey.

[5] Gum Tragacanth, Fisher Scientific Company, Pittsburgh, Pennsylvania.

monolayer. It was prepared by mixing equal quantities of double strength MEM and gum tragacanth solution and adding 2% fetal bovine serum and antibiotics at the standard concentrations described above.

Trypsin-Versene Solution was used to remove cell monolayers from the substrate on which they had grown during routine cell passaging. Its formula is given in Table 3 at the end of this section.

Cell Culture

Madin-Darby bovine kidney (MDBK) cells were used exclusively in this study. Their true passage number was not known as the previous history of the line was not available, but the experiments described here were conducted using cells within a range of thirty serial passages.

The cell line was maintained by growth in single-use 250 ml plastic tissue culture flasks[1] in the standard growth medium without antibiotics, being incubated at 37°C in a humidified incubator with a 5% carbon dioxide atmosphere. The caps of the flasks were screwed on lightly so as to permit gaseous exchange. With such a regimen the cells would grow sufficiently well to allow twice weekly serial passages with preparation of three flasks from one flask of cell culture. After pouring off the old medium the cells were removed from the fully-grown flasks in a matter of minutes using trypsin-versene solution, which in turn was separated from the detached cells which were sedimented as a pellet by gentle centrifugation at 600 revolutions per minute for 10 minutes. The waste

---

[1] Corning Glass works, Corning, New York.

trypsin-versene was then poured off and the cells were resuspended in fresh growth medium at a dilution three times greater than hitherto, and dispensed into new flasks for continuation of the growth cycle or into single-use plastic cell culture plates.[1] When cell cultures in plates had formed confluent monolayers they were used for viral assay using the plaque technique (see later). Antibiotics were used in these terminal passages.

Virus

The virus used throughout the experiments was the seventh laboratory passage of a strain which had been obtained from pigs in a field outbreak in Iowa studied by G. W. Beran (Department of Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa) in 1974. It was known variously as S62/26, the "Wilson" strain and the "Iowa" strain. While accepting that such judgments are subjective, it had been assessed in the field as being moderately virulent. It had been cloned twice in the laboratory before use in this study (courtesy of Drs. Maré and Platt).

Stock virus was prepared by heavily inoculating cell cultures in flasks in which the monolayers were just on the point of becoming confluent with a large amount of virus and incubating at 37°C for a little over 24 hours until most of the cells showed cytopathic effect. The fluid in the flasks was then twice frozen rapidly to -90°C and thawed rapidly to 4°C in an ice bath to disrupt the remaining cells to promote liberation of virus, centrifuged lightly to remove cellular debris, and

[1] Lux Plates, Lux Scientific Corporation, Newbury Park, California.

filtered through a methyl cellulose membrane filter of pore diameter 450 nm. This latter procedure is practiced to remove from the fluid any clumps of virus, while allowing the passage of the single virions of size 150-170 nm. The harvested virus suspensions were pooled to insure uniformity. The pH of the pool was then adjusted to 6.8, and it was aliquoted in 1 ml portions in small screw cap vials and stored at -90°C.

Sterilization Processes and Cleansing of Glassware

Infected materials and equipment, whether for disposal or reuse, were routinely autoclaved for 45 minutes at a pressure of 20 pounds per square inch before further handling, excepting for infected reusable pipettes, which were disinfected by immersion in acetic acid solution (10% glacial in water) in upright pipette jars.

Detergents have a deleterious effect upon cell cultures. Glassware used in this study was washed in a nonsudsing detergent,[1] rinsed four times in tap water, rinsed eight times in deionized water and air- or oven-dried before wrapping and sterilizing by autoclaving at a pressure of 20 pounds per square inch for 20 minutes. Stubborn strains were removed from glassware with a cleansing solution, the formula of which is given in Table 4.

Viral Assay by Plaque Counting Technique

When the titers of virus-containing suspensions were to be measured, the test materials were randomized and placed in an ice bath. A serial dilution of each was then made in turn by transferring 0.4 ml of solution

[1] "Disperse", Scientific Products, McGaw Park, Illinois.

into 3.6 ml of saline G at pH 7.0 with antibiotics to give a dilution of one in ten, the procedure being repeated stepwise to a degree depending upon the anticipated amount of virus in the suspension.

Meanwhile the cell culture plates were also randomized and in turn the nutrient medium was aspirated from their wells and replaced in each by one ml of each of the serial decimal dilutions of the virus. Each plate was then incubated for one hour at 37°C in an atmosphere of 5% carbon dioxide in a gassed incubator[1] to allow adsorption of the virus. Then the test solution was removed and replaced with 2 ml overlay medium, the plate again being incubated under the same conditions, but this time for 48 hours to allow virus multiplication and cell destruction in the form of plaques under the overlay. The virus was then inactivated and the cell culture cells fixed by the addition of 2 ml of an approximately 6% solution (weight/volume) of formalin in water. After 20 minutes the formalin-overlay mixture was poured off and the plaques made easily visible by staining with 2% aqueous crystal violet solution for ten minutes. The plates were washed and dried and the plaques counted.

In successful experiments the plaques were consistently about one and one-half millimeters in diameter and clearly visible against a dense, dark blue background formed by the portion of the cell sheet undamaged by virus. Experiments in which this was not so were repeated.

At all stages in the procedure a single operator performed the whole of a single task. The usual procedure was for one operator to make the

---

[1]Forma Scientific Inc., Marietta, Ohio.

dilution series while a second operator followed behind inoculating the plates. Plaque counting was performed by a single experienced operator who was not responsible for the design of the experiment. Where possible, wells with around 200 plaques each were counted. The particular type of plates used here contained eight wells, and it was the practice to use two wells at each virus dilution, thereby allowing the titration of four separate dilutions on each plate. During the calculation of the end points, the counts of the two equivalent wells were averaged.

Adjustment of pH of Suspensions Containing Virus

During these experiments, all virus was maintained in saline G at test pH levels, adjusted using 0.1 N hydrochloric acid and 0.1 N sodium hydroxide. pH was measured using an electronic pH[1] meter, the calibration of which was checked before each use against stock solutions of buffer of known pH.[2] Whenever possible, samples which were to be compared after varying treatments were prepared as one stock which was then divided into aliquots to insure uniformity. It is difficult to adjust the pH of small quantities of suspension and the time taken to effect the adjustment may vary considerably between samples depending upon whether the correct amount of acid or alkali to add is judged successfully on the first attempt, or whether repeated adjustments are required. During the procedure it is quite easy to inadvertently adjust the pH to unintended values before the final correction. If therefore, virus is present in the

---

[1]Coleman Instruments, Maywood, Illinois.

[2]"pH Buffar", Mallinckrodt, St. Louis, Missouri.

suspension at the time, it may be exposed to an unintended (and possibly unknown) pH for a period of time. To avoid this problem, stock virus was kept at pH 7.0, and when adjustment of pH was necessary, a small sample was taken and injected into a much larger sample (dilution 1-10) of saline G at a pH a little further removed from pH 7.0 than the required final pH. This resulted in an immediate adjustment of the pH of the virus-containing suspension to a value approximately that which was required. The pH of this new (mixed) suspension was then measured and the experiment conducted using that value, with no attempt to further adjust the pH being made. In practice, a final pH was usually obtained which was within 0.1 of a pH unit of that which was intended. These procedures were routinely conducted in icebaths, except when (see later) there was a particular reason for not doing so.

Temperature Adjustment

Virus suspensions were maintained for varying periods of time at temperatures ranging from $-90^{\circ}C$ to $37^{\circ}C$ in standard equipment, freezers[1] and refrigerators[2] for the low temperatures, incubators for the high temperatures,[3] and refrigerated-incubators[4] for the intermediate range of temperatures close to room temperature where neither refrigerator nor incubator could be relied upon to maintain the required temperatures.

---

[1]Revco Inc., Deerfield, Michigan.

[2]Sears Coldspot Refrigerators, Sears Roebuck and Co., Chicago, Ill.

[3]GCA/Precision Scientific, Chicago, Illinois.

[4]Hotpack, Philadelphia, Pennsylvania.

For short-term use, ice baths and water baths were also used. The former were used routinely at all times during virus-handling procedures such as adjustment of pH or the preparation of virus dilutions.

When very rapid temperature adjustment was required, as in the "0 hour" samples at a particular temperature, the same technique was used as for the rapid adjustment of pH described previously. One part virus suspension at ice bath temperature was injected into nine parts Saline G at the required temperature held in a tube in a water bath, and the dilution effect together with the high latent heat of the containing-tube and that of the surrounding water rapidly adjusted the temperature to that required. In practice temperature and pH adjustment were carried out simultaneously by this procedure for the "0 hour" samples at various pH. The equilibration of the temperature was monitored on a dummy tube.

When fluctuating temperatures were required, these were obtained by various ways. For temperatures above freezing point the virus solution was placed in test tubes in a rack which was moved between an ice bath at 4°C and a water bath at 37°C. A dummy tube containing the same amount of Saline G and a thermometer was included in the rack to monitor the equilibration of the temperature before transfer to the other container. By this technique, ten complete temperature cycles could be obtained in one hour. For temperatures below freezing, fluctuation was not so easily controlled with the equipment at hand, as it was not possible to measure the temperature inside the frozen aliquots. Frozen virus-suspension aliquots were kept at the required ambient temperatures for periods amply sufficient to ensure temperature equilibration. When

changing the temperature between 4°C and -90°C, an ice bath was used for the higher temperature and a precooled bath of acetone in the freezer for the lower temperature. This produced rapid heat exchange and rapid freezing and thawing. When changing the temperature between -90°C and -13°C, the aliquots were placed in respectively the Revco freezer and the freezing compartment of a refrigerator for apporximately 12 hours to allow equilibration.

Drying Virus

Virus was dried by two methods. In one, 3 ml samples of virus-suspension at various pH levels were placed in open glass Petri dishes at the test temperature. At 37°C and a relative humidity of 30%, complete drying was achieved in two hours. At 15°C and 25°C and a relative humidity of 40-50% drying was achieved overnight. In all cases dried sediments were reconstituted by adding 3 ml of distilled water to the Petri dishes and thoroughly removed from the glass by alternately withdrawing the suspending fluid into a pipette and blowing it back out over the glass six times. During the drying procedure, all samples had parallel aliquots in sealed tubes placed alongside them following the same temperature sequence to act as undried controls.

In addition to drying virus on glass, aliquots were also dried on sheets of gelatin. These were prepared by dissolving the proprietary[1] powered gelatin in hot water and pouring the solution on to aluminum foil, where it was allowed to dry for 1-2 days before removal and

[1] Difco Laboratories, Detroit, Michigan.

division into small sheets about 1 cm square and less than 0.5 mm thick. Small aliquots of virus suspension, 0.1 ml, were placed on the gelatin sheets and these were then placed in open Petri dishes to dry at the appropriate temperature. The gelatin was then placed in 4.9 mm Saline G at 4°C and left 48 hours to dissolve and thereby resuspend any surviving virus. Control suspensions on gelatin followed the same regimen except that they were not allowed to dry but were instead immediately placed in the Saline G.

Ultraviolet Light

The effect of ultraviolet (U/V) light was studied using a General Electric U/V germicidal tube (G15T8)[1] of 15 watts held approximately 30 inches above thin layers of virus suspension at various pH levels in open glass Petri dishes. Three milliliters of solution were put into each Petri dish and as these were 8.5 cms in internal diameter it formed a layer about 0.5 mm thick. Virus suspensions were exposed to the U/V light for periods of 20, 40 and 60 minutes, the two shorter intervals being obtained by covering the dishes with thick aluminum foil at the appropriate time and leaving them under the U/V light. All the Petri dishes, therefore, remained in the vicinity of the light for 60 minutes and they were accompanied for this period by a control suspension in a Petri dish which was covered with aluminum foil for the whole time. Additional controls were provided by a further set of uncovered Petri dishes laid on the bench and at the same ambient temperature (22°C), but

[1] General Electric, Schnectady, New York.

not exposed to the U/V light or to any ozone which may have been generated by it. The experimental units therefore comprised at each test pH levels, the covered control held near the U/V source for 60 minutes, the 3 test samples exposed to the light for 20, 40 and 60 minutes respectively but held near the U/V source for the whole 60 minutes, and a further control not exposed to the U/V light in any way, but open to the atmosphere for 60 minutes. Preliminary experiments had shown that at the ambient temperature a loss of volume of 0.6 ml due to evaporation could be expected in 1 hour. To allow for this, at the start of each 20 minutes period, each Petri dish, whether exposed to U/V light or not, which was to remain open received 0.2 ml of distilled water. In addition to the 5 test samples described here, a sample of the starting virus solution was kept at each test pH in closed tubes in an ice bath for the whole of the period of the experiment and then titrated in parallel as a comparison.

Table 1. Preparation of Minimum Essential Medium (MEM)

Component	Amount	Comments
Proprietary MEM[a] Powder (Earle's Salts, L-glutamine, Nonessential Amino Acids)	One packet of appropriate size Gibco #410-1500	
Triple distilled water	Approximately 4,900 ml	Dissolve at room temperature with gentle stirring
Sodium bicarbonate ($NaHCO_3$)	22 grams	
Deionized water	Make up to 5,000 ml	

Adjust pH using 1N hydrochloric acid or 1N sodium hydroxide to 0.2-0.3 below desired final pH.

Sterilize by filtering through a Millipore filter[b] of pore diameter 200 nm.

The pH will rise 0.2-0.3. This procedure will produce double-strength MEM. Prepare single-strength MEM by mixing with an equal quantity of sterile triple distilled water.

[a]Grand Island Biological Company, Grand Island, New York.

[b]Millipore Corporation, Bedford, Massachusetts.

Table 2. Preparation of Saline G

Components	Amount	Comments
Dextrose	1.1 grams	
Sodium chloride ($NaCl$)	8.0 grams	
Potassium chloride (KCl)	0.4 grams	
Potassium phosphate (KH_2PO_4)	0.15 grams	
Sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$)	0.29 grams	
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.15 grams	
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.016 grams	
Lactalbumin hydrolysate	1.0 grams	
Phenol Red	0.0012 grams	
Triple distilled water	To 1 liter	Dissolve with gentle stirring at room temperature

Adjust pH using 1N hydrochloric acid or 1N sodium hydroxide to 0.2-0.3 below described final pH

Sterilized by filtering through a Millipore filter[a] of pore diameter 200 nm.

pH will rise 0.2-0.3.

[a]Millipore Corporation, Bedford, Massachusetts.

Table 3. Preparation of trypsin-versene solution

Components	Amount	Comments
Sodium chloride (NaCl)	16.0 grams	
Potassium chloride (KCl)	0.8 grams	
Sodium bicarbonate ($NaHCO_3$)	1.4 grams	
Dextrose	2.0 grams	
Versene[a]	0.4 grams	
Phenol Red 1% solution	2 ml	
Triple distilled water	Approximately 180 ml	Mix and dissolve with gentle stirring.
Trypsin[b] 1:250	1.0 grams	Derived from hog pancreas. Porcine parvovirus tested. Heat solution to 37°C before adding trypsin. It will take approximately one hour to dissolve
Triple distilled water	Make up to 200 ml	

Sterilize after cooling by filtering through a Millipore filter[c] of pore diameter 200 nm.

This procedure will produce X10 Trypsin-Versene solution.

Dilute 1:10 in sterile triple distilled water before use.

[a]Disodium ethylene diamine tetra-acetate, Nutritional Biochemical Corporation, Cleveland, Ohio.

[b]Gibco Grand Island Biological Company, Grand Island, New York.

[c]Millipore Corporation, Bedford, Massachusetts.

Table 4. Preparation of glassware cleansing solution

Component	Amount	Comments
Sodium dichromate ($Na_2Cr_2O_7 . 2H_2O$	120 grams	
Deionized water	1,000 ml	Dissolve with gentle stirring.
Concentrated sulfuric acid (H_2SO_4)	1,600 ml	Mix, taking care to avoid splashing.

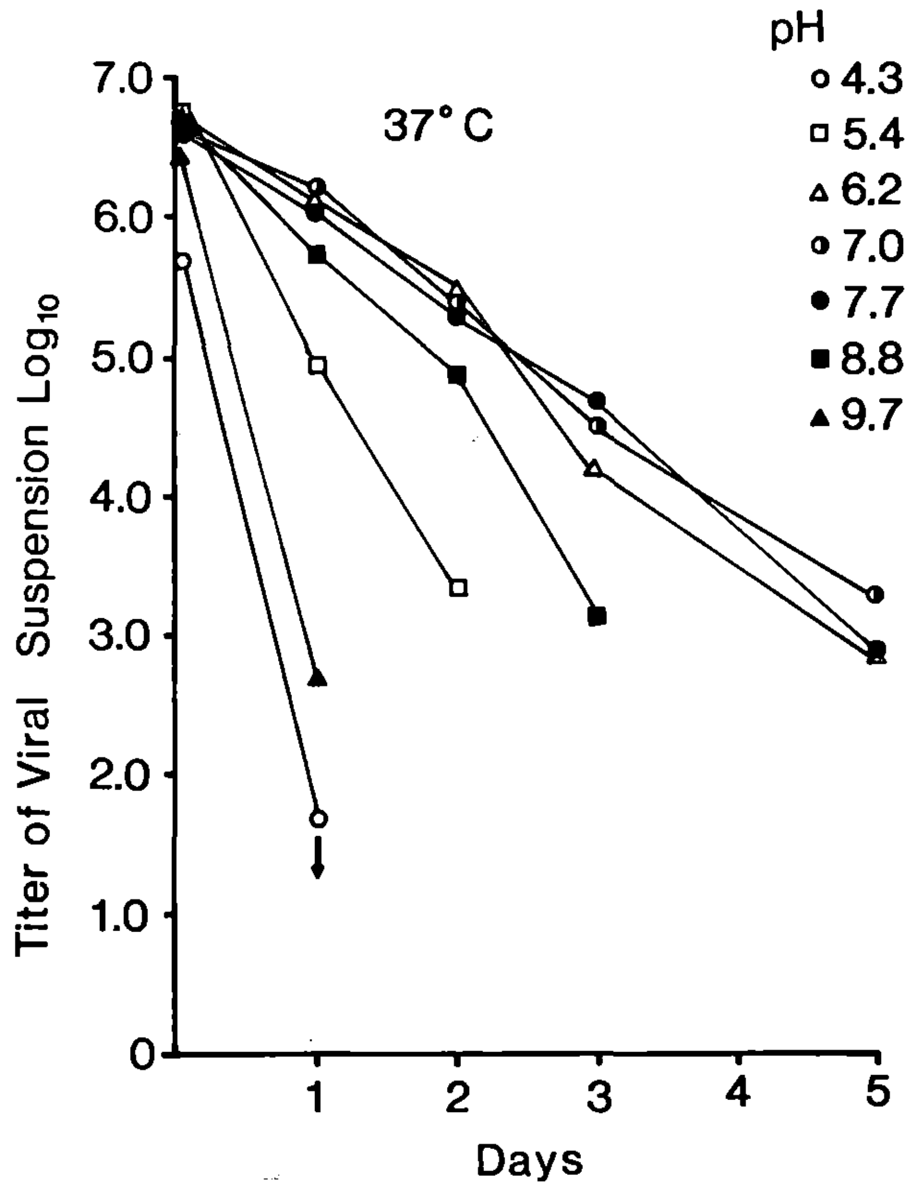
Warning - This solution is highly corrosive and should be handled with care.

RESULTS

The Effect of Constant Temperature and Various pH Levels Upon the Survival of Virus

The first group of experiments in this series investigated the survival of virus held at several constant temperatures above the freezing point using virus suspensions at a range of pH levels from 4.3 to 9.7. The temperatures used were 37°C, 25°C, 17°C and 4°C. The titers of virus surviving were plotted in $\log_{10}$ units against time in Figures 1, 3, 5 and 7 respectively. They showed a straight line relationship in each case. From these figures, the rate of inactivation of each virus suspension at each tested pH level was calculated and plotted against pH level at the various temperatures, as shown in Figures 2, 4, 6 and 8. This produced graphs in which the pH levels optimal for virus survival could readily be distinguished, and in which the minimum possible titer loss at that temperature could readily be measured as the distance between the lowest point of the curve and the abscissa. These minimum distances were plotted against temperature in Figure 9, obtaining a curve from which may be read the minimum loss of titer which a virus suspension at optimal pH level will lose at various temperatures. Virus suspensions not at optimal pH level will lose titer at a greater rate.

Figure 1. The effect of a constant temperature of 37°C upon the titer of suspensions of Aujeszky's Disease virus held at various pH levels



37° C
pH
4.3
5.4
6.2
7.0
7.7
8.8
9.7
Titer of Viral Suspension Log10
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1
2
3
4
5
Days

Figure 2. The rate of loss of titer of Aujeszky's Disease virus suspensions at various pH levels held at a constant temperature of 37°C

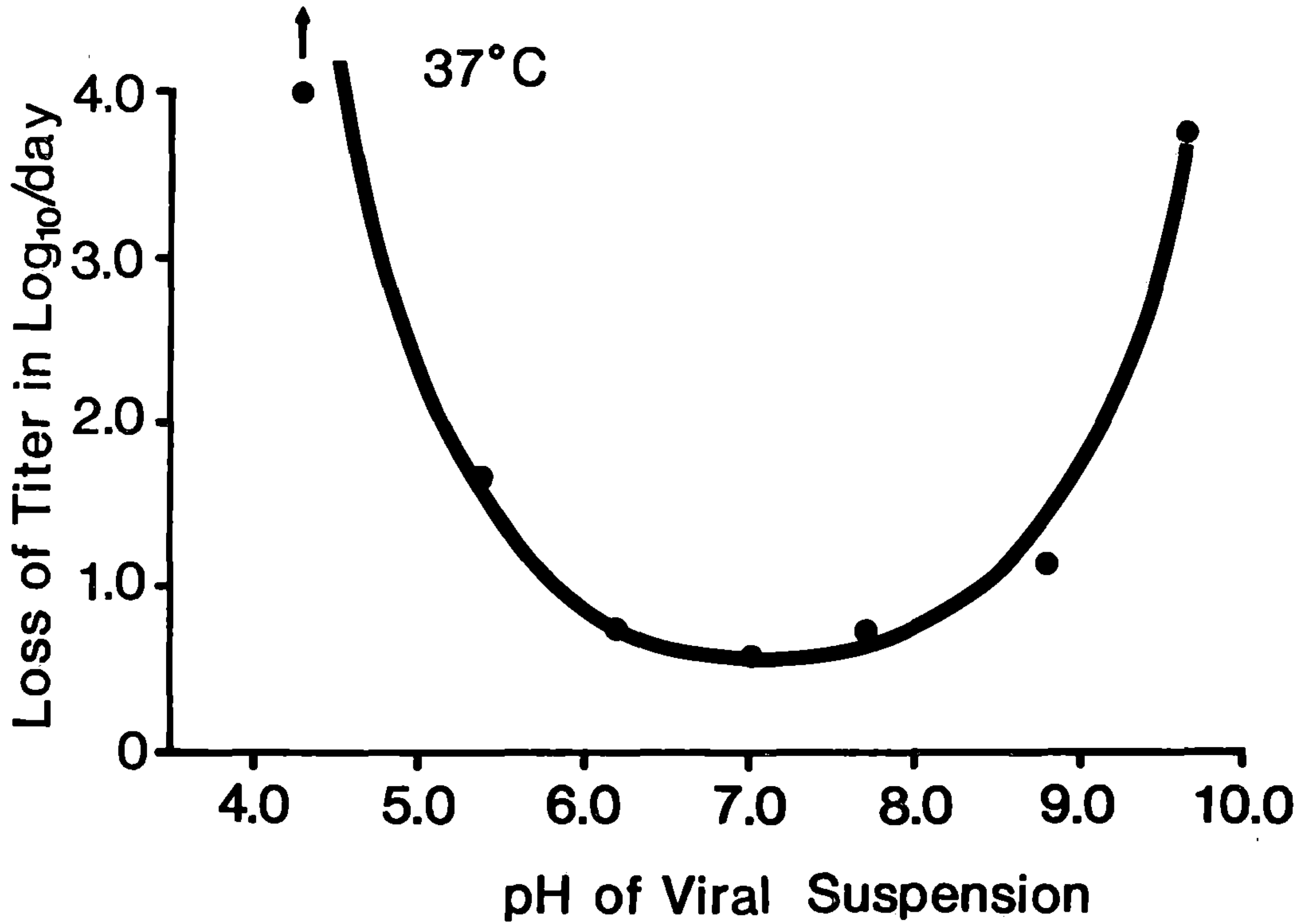
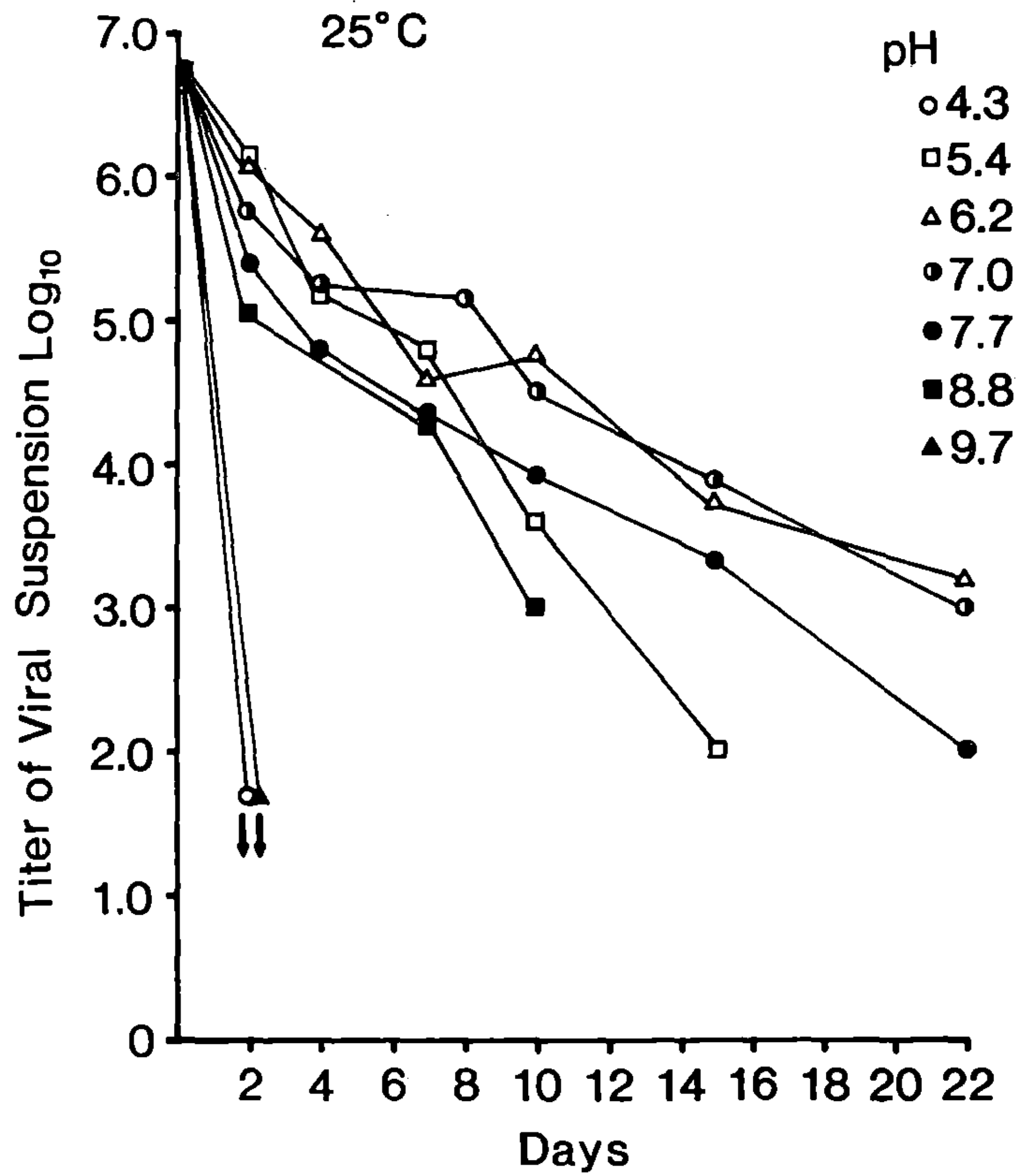
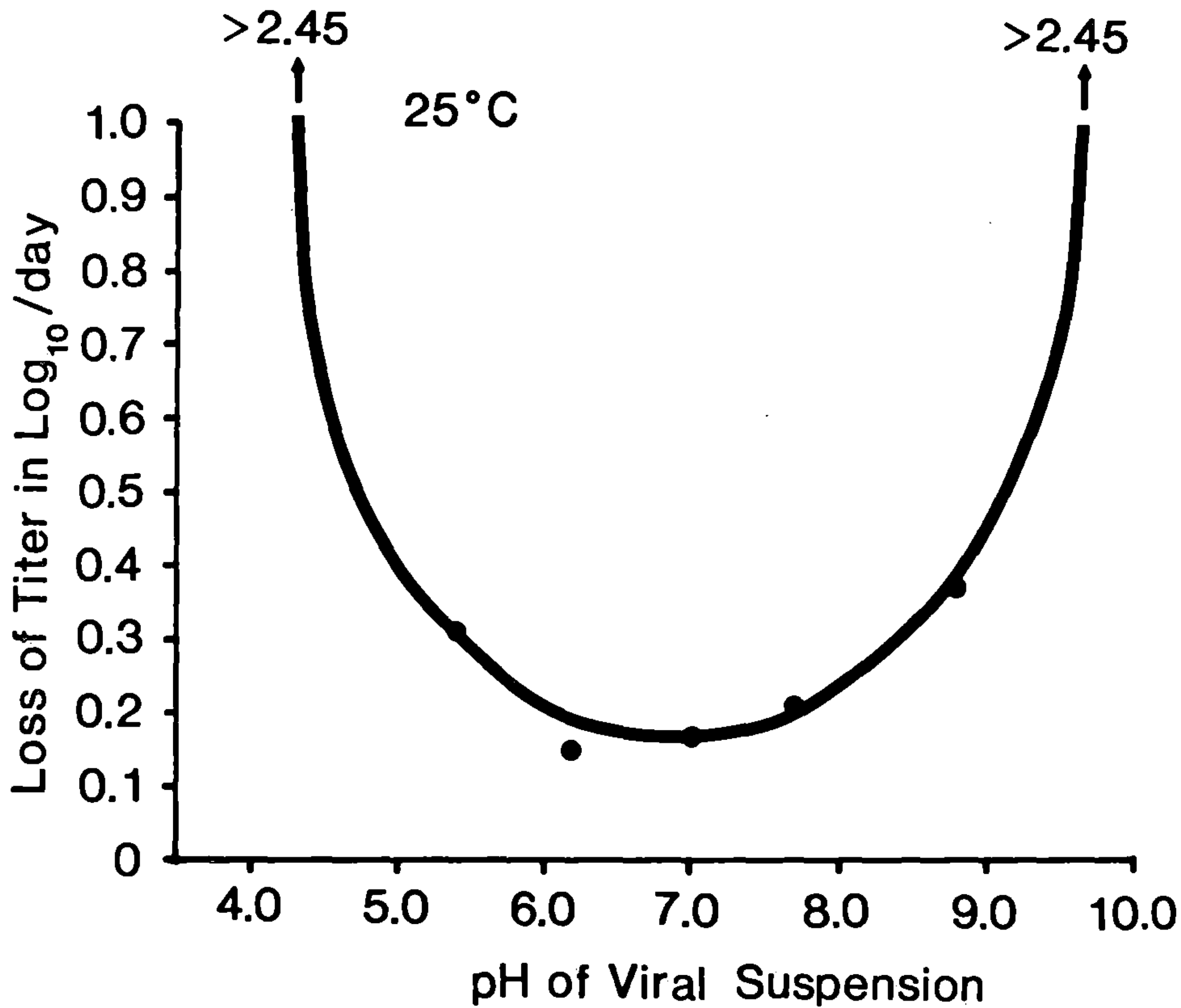

37°C
Loss of Titer in Log10/day
4.0
3.0
2.0
1.0
0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
pH of Viral Suspension

Figure 3. The effect of a constant temperature of 25°C upon the titer of suspensions of Aujeszky's Disease virus held at various pH levels



25°C
pH
4.3
5.4
6.2
7.0
7.7
8.8
9.7
Titer of Viral Suspension Log10
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
2 4 6 8 10 12 14 16 18 20 22
Days

Figure 4. The rate of loss of titer of Aujeszky's Disease virus suspensions at various pH levels held at a constant temperature of 25°C



>2.45
>2.45
25°C
Loss of Titer in Log10 /day
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
pH of Viral Suspension

Figure 5. The effect of a constant temperature of 17°C upon the titer of suspensions of Aujeszky's Disease virus held at various pH levels

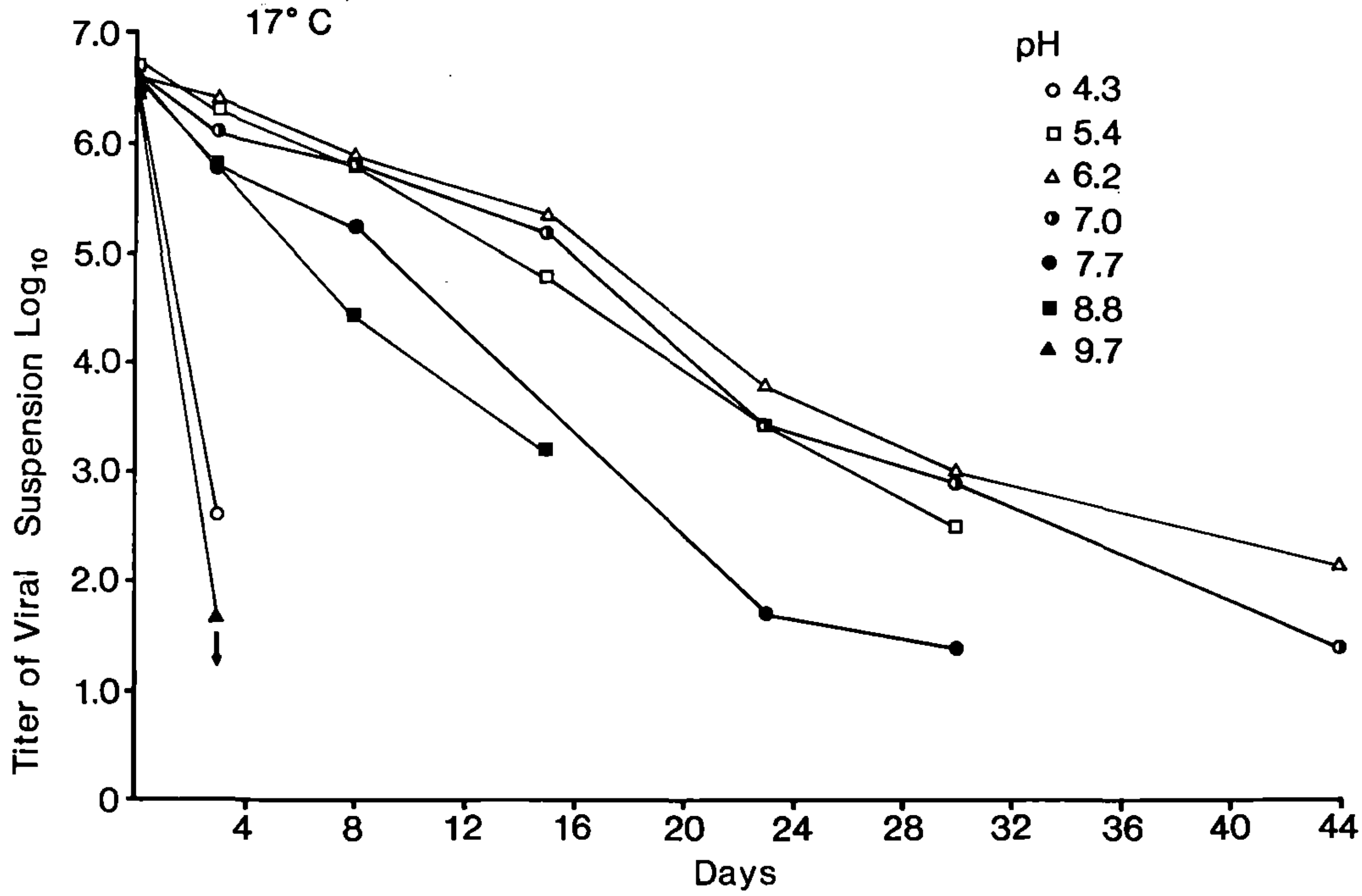
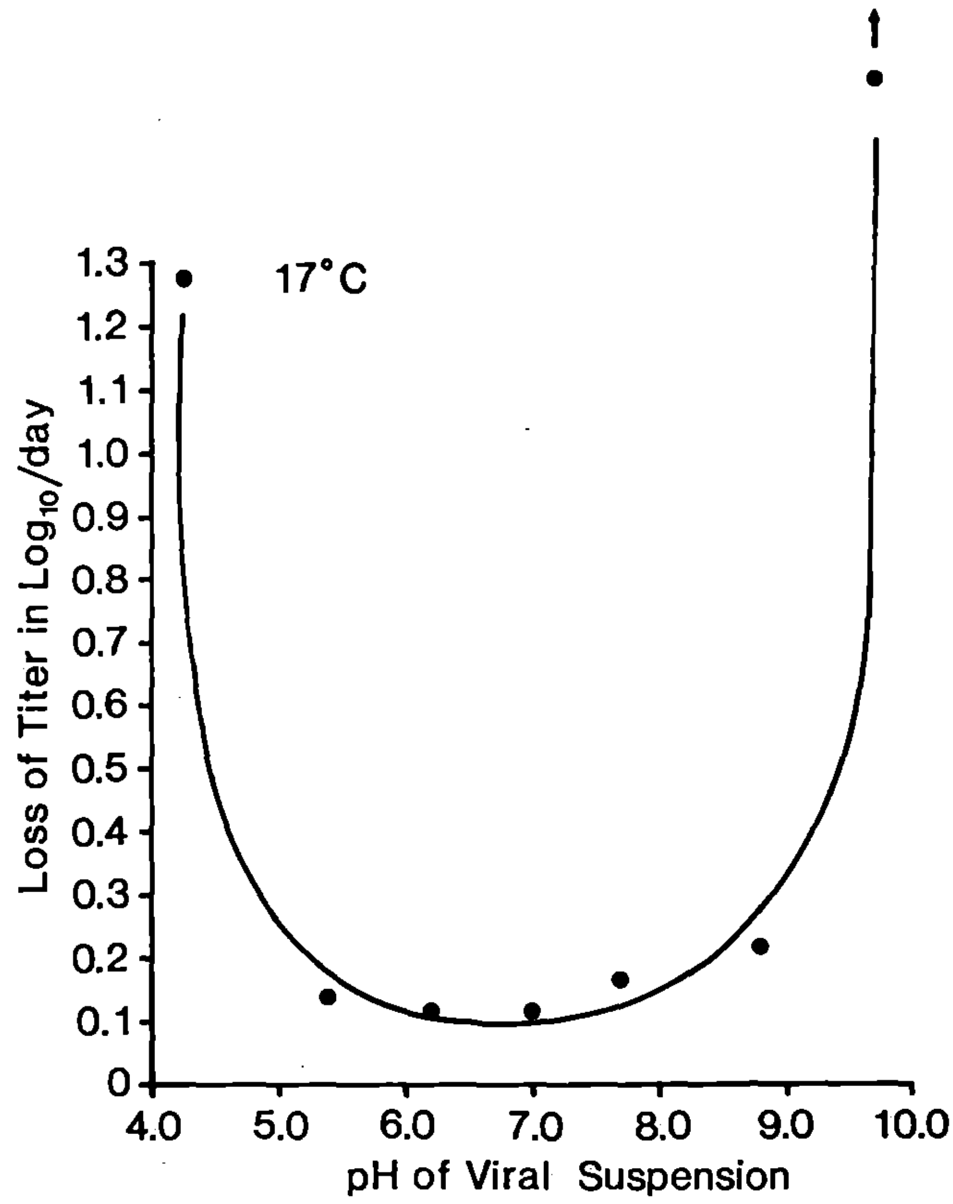
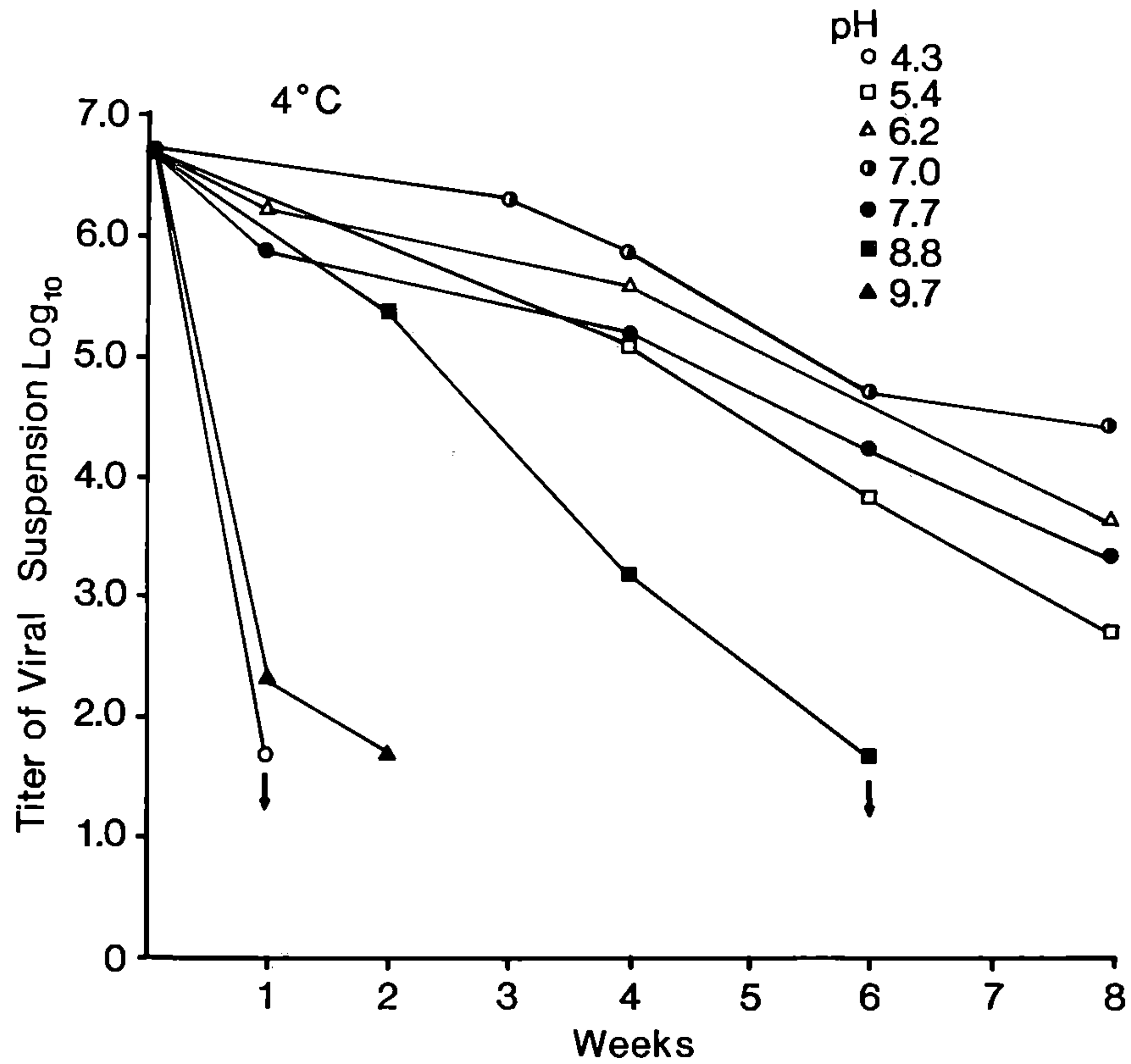

17° C
pH
4.3
5.4
6.2
7.0
7.7
8.8
9.7
Titer of Viral Suspension Log_{10}
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
4
8
12
16
20
24
28
32
36
40
44
Days

Figure 6. The rate of loss of titer of Aujeszky's Disease virus suspensions at various pH levels held at a constant temperature of 17°C



17°C
Loss of Titer in Log_{10}/day
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
pH of Viral Suspension

Figure 7. The effect of a constant temperature of 4°C upon the titer of suspensions of Aujeszky's Disease virus held at various pH levels



4°C
pH
4.3
5.4
6.2
7.0
7.7
8.8
9.7
Titer of Viral Suspension Log_{10}
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
1
2
3
4
5
6
7
8
Weeks

Figure 8. The rate of loss of titer of Aujeszky's Disease virus suspensions at various pH levels held at a constant temperature of 4°C

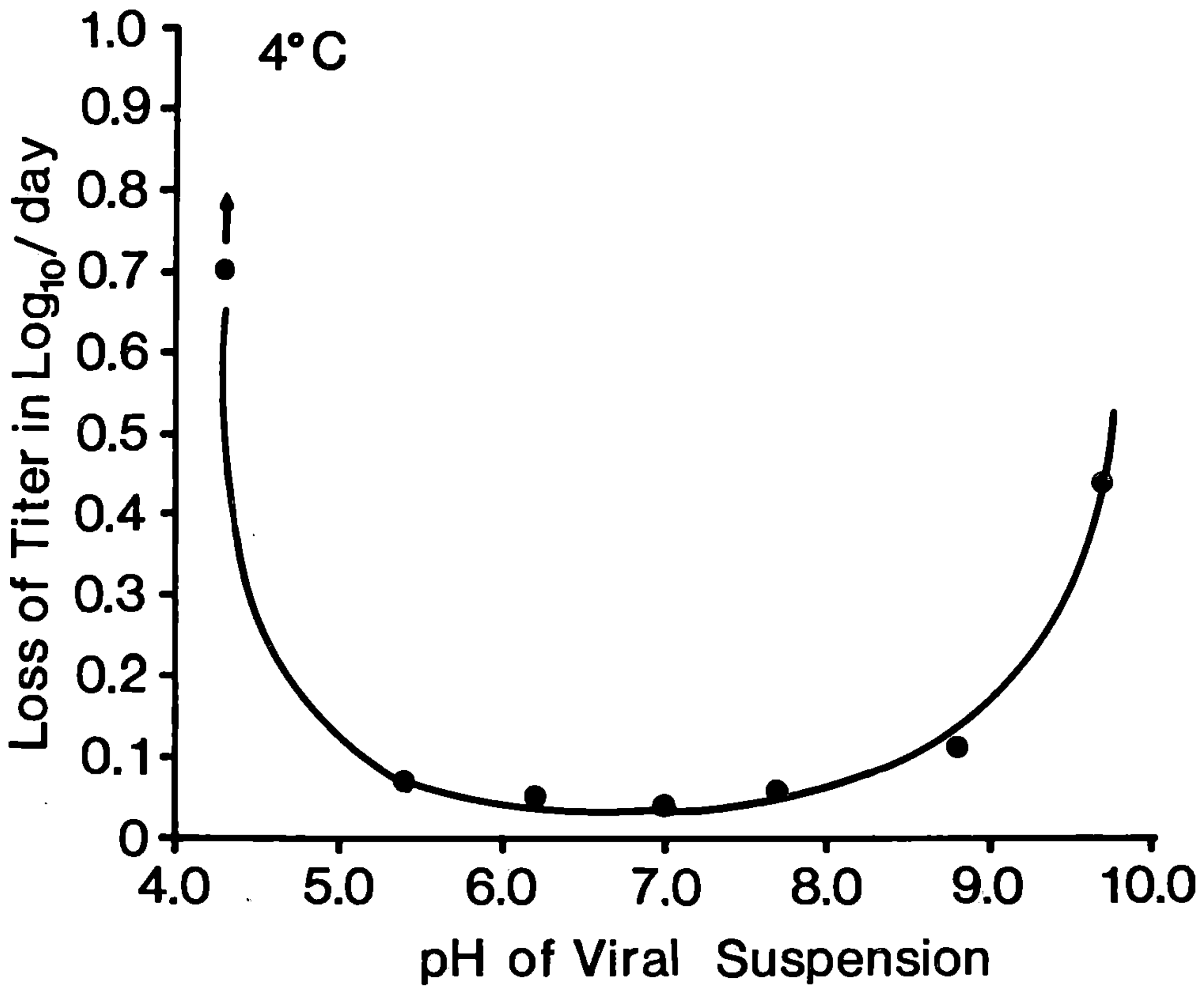
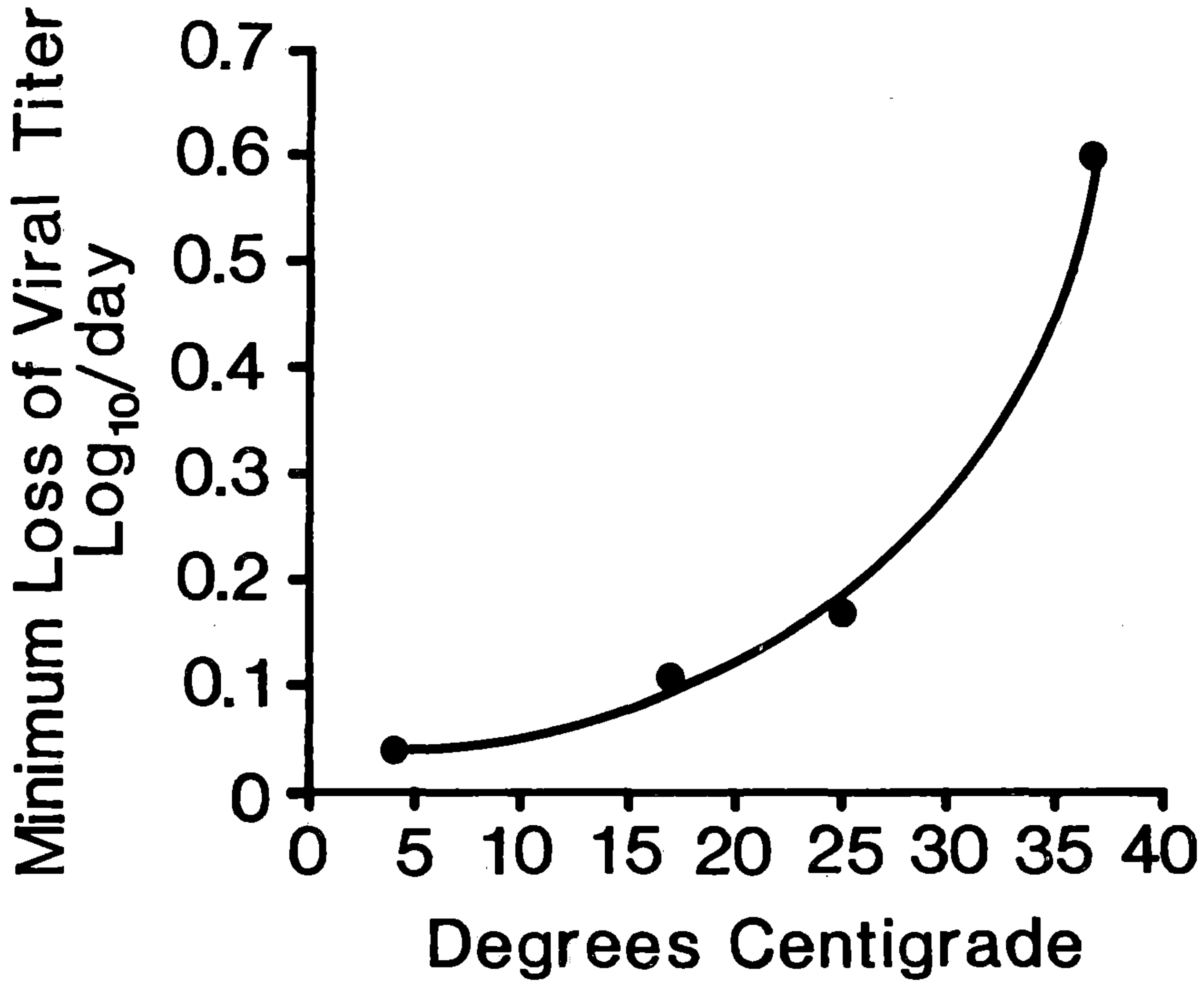

4°C
Loss of Titer in Log_{10}/ day
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
pH of Viral Suspension

Figure 9. The minimum rate of loss of titer of Aujeszky's Disease virus suspensions held at optimal levels of pH at different constant temperatures



Minimum Loss of Viral Titer
Log10/day
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
Degrees Centigrade

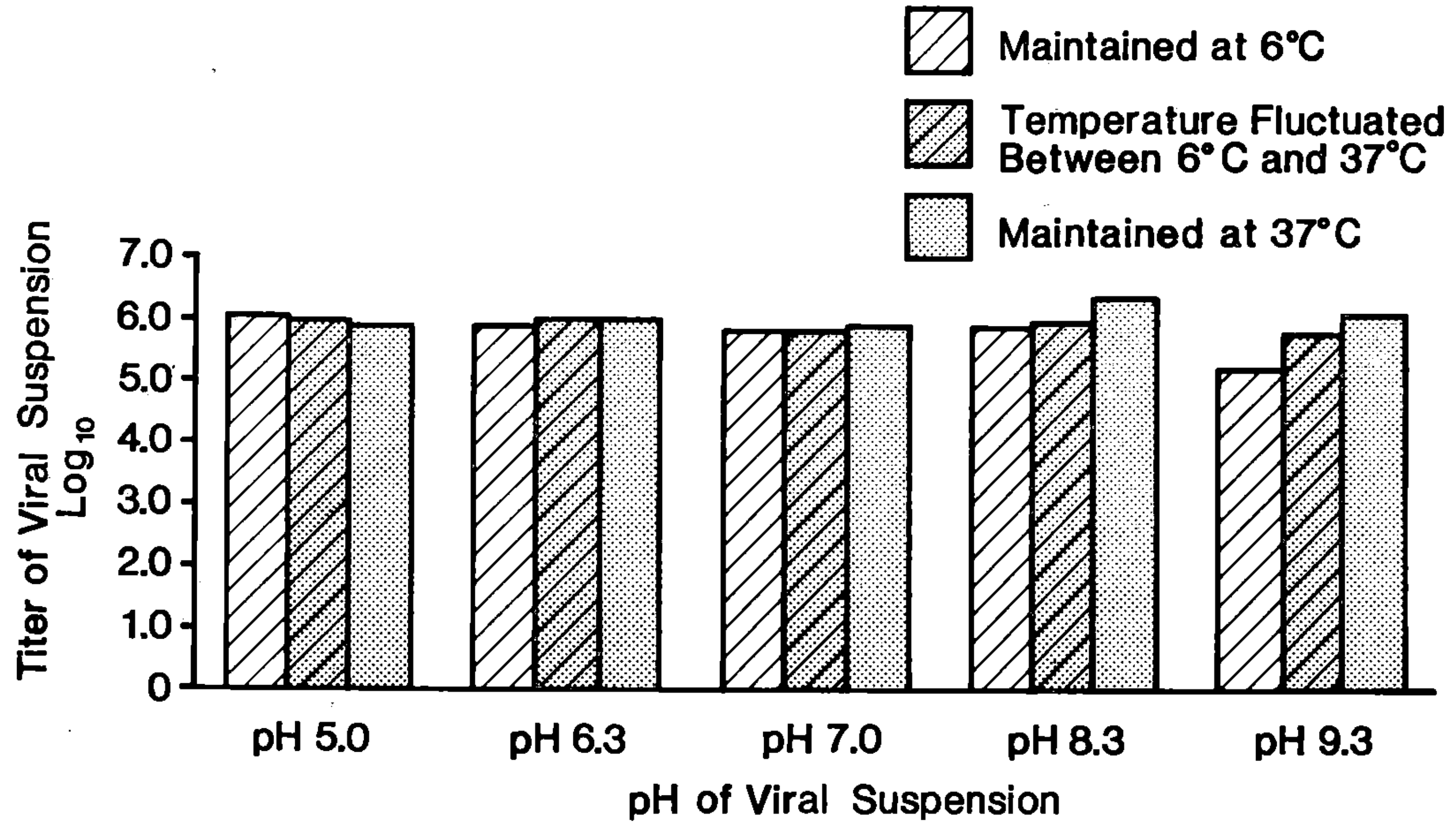
The Effect of Temperature Fluctuation and pH Level Upon the Survival of Virus

The second group of experiments in this series investigated the survival of virus held at fluctuating temperatures using virus suspensions at a range of pH levels. In Figure 10 the titers of virus suspensions which were alternately raised and lowered in temperature 10 times between 37°C and 6°C are compared with those of similar virus suspensions held at constant temperatures of 6°C and 37°C. In Figure 11 the titers of virus suspensions which were frozen to -90°C and thawed once, twice and three times are compared with those of virus suspensions which were not frozen at all. Figure 12 shows the effect upon the titers of virus suspensions at various pH levels subjected to three temperature fluctuations in the frozen state between -90°C and -13°C.

Storage of Virus Suspensions by Freezing

The third group of experiments in this series investigated the factors affecting the survival of virus under conditions of storage by freezing using virus suspensions at various pH levels. Figure 13 shows the effect of freezing at -90°C for three days compared with freezing at -13°C for the same period upon the titers of virus suspensions. Figure 14 shows the loss of titer which was found at various pH levels when virus suspensions were frozen to -90°C and thawed as rapidly as possible without storage at the low temperature. Figure 15 depicts the same information in graphic form to emphasize the deleterious effect of the higher pH levels. Figure 16 shows the effect upon the titer of long-term storage of virus at -90°C at various pH levels.

Figure 10. The effect of ten temperature fluctuations between 6°C and 37°C, compared with the effect of constant temperatures of 6°C and 37°C, upon the titer of Aujeszky's Disease virus suspensions held at various pH levels



Maintained at 6°C
Temperature Fluctuated Between 6° C and 37°C
Maintained at 37°C
Titer of Viral Suspension Log10
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
pH 5.0
pH 6.3
pH 7.0
pH 8.3
pH 9.3
pH of Viral Suspension

Figure 11. The effect of freezing and thawing once, twice and three times, compared with the effect of not freezing, upon the titer of Aujeszky's Disease virus suspensions held at various pH levels.

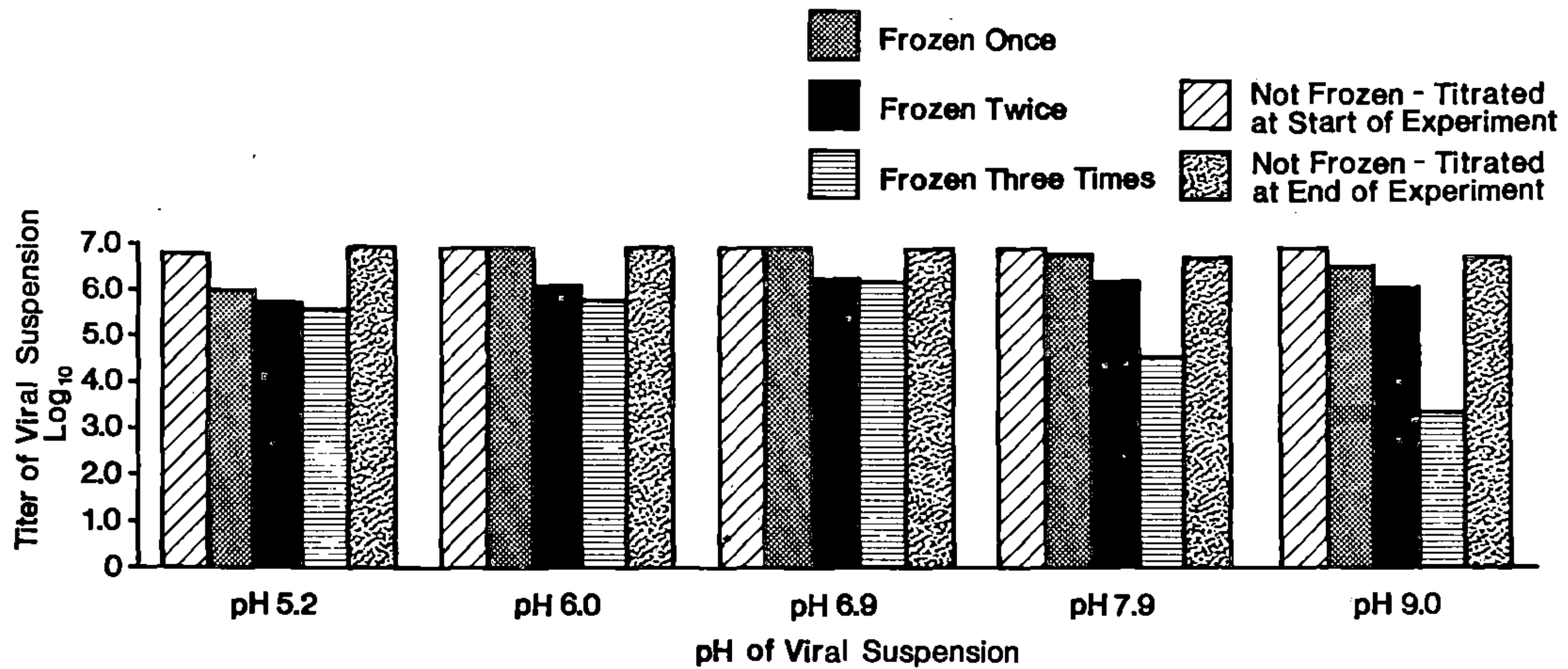
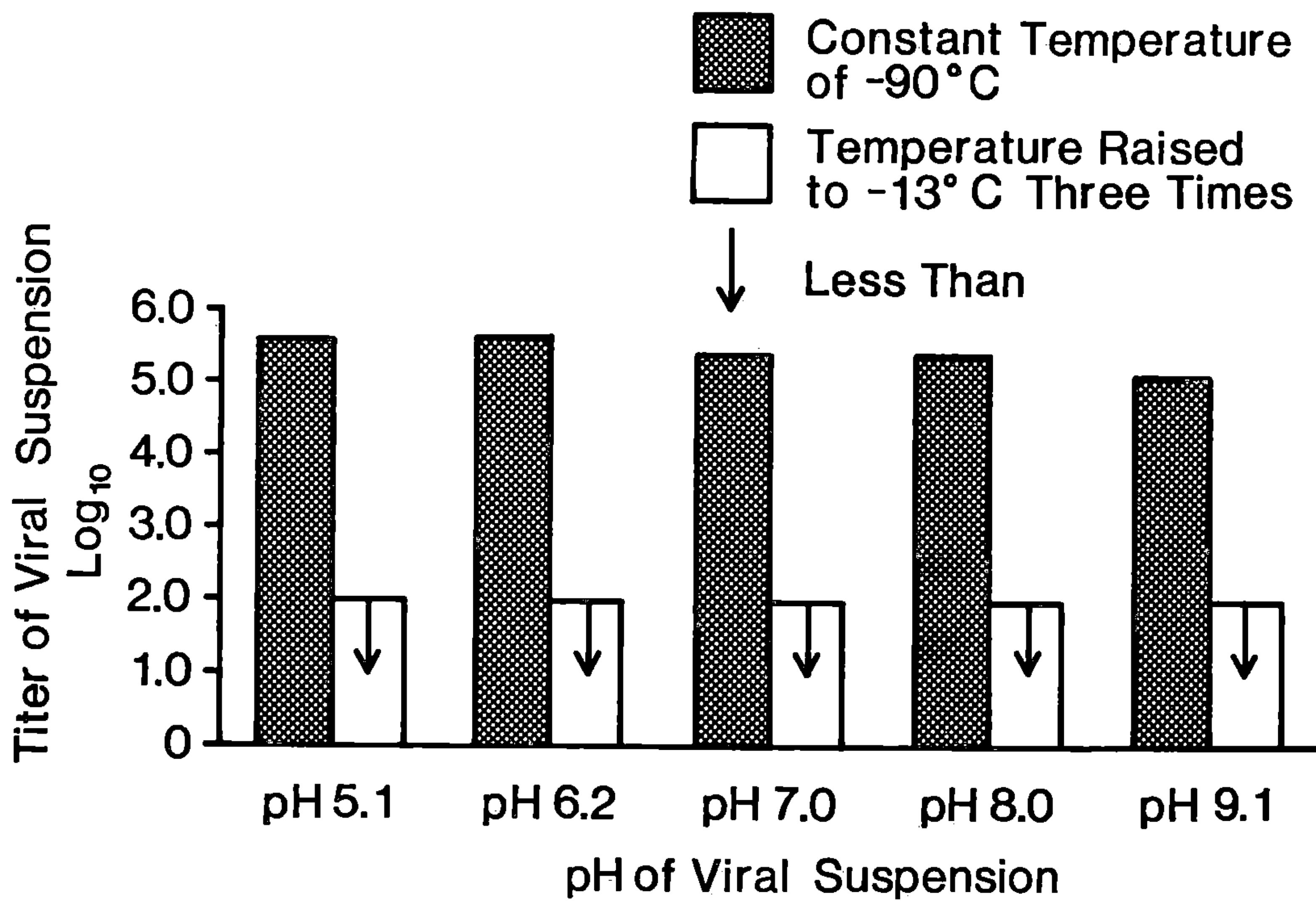

Frozen Once
Frozen Twice
Frozen Three Times
Not Frozen - Titrated at Start of Experiment
Not Frozen - Titrated at End of Experiment
Titer of Viral Suspension Log_{10}
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
pH 5.2
pH 6.0
pH 6.9
pH 7.9
pH 9.0
pH of Viral Suspension

Figure 12. The effect of three temperature fluctuations between -90°C and -13°C, compared with the effect of a constant temperature of -90°C, upon the titer of Aujeszky's Disease virus suspensions held at various pH levels



Constant Temperature of -90°C
Temperature Raised to -13° C Three Times
Less Than
Titer of Viral Suspension Log10
6.0
5.0
4.0
3.0
2.0
1.0
0
pH 5.1
pH 6.2
pH 7.0
pH 8.0
pH 9.1
pH of Viral Suspension

Figure 13. The effect of storage at -90°C, compared with the effect of storage at -13°C, upon the titer of Aujeszky's Disease virus suspensions at various pH levels

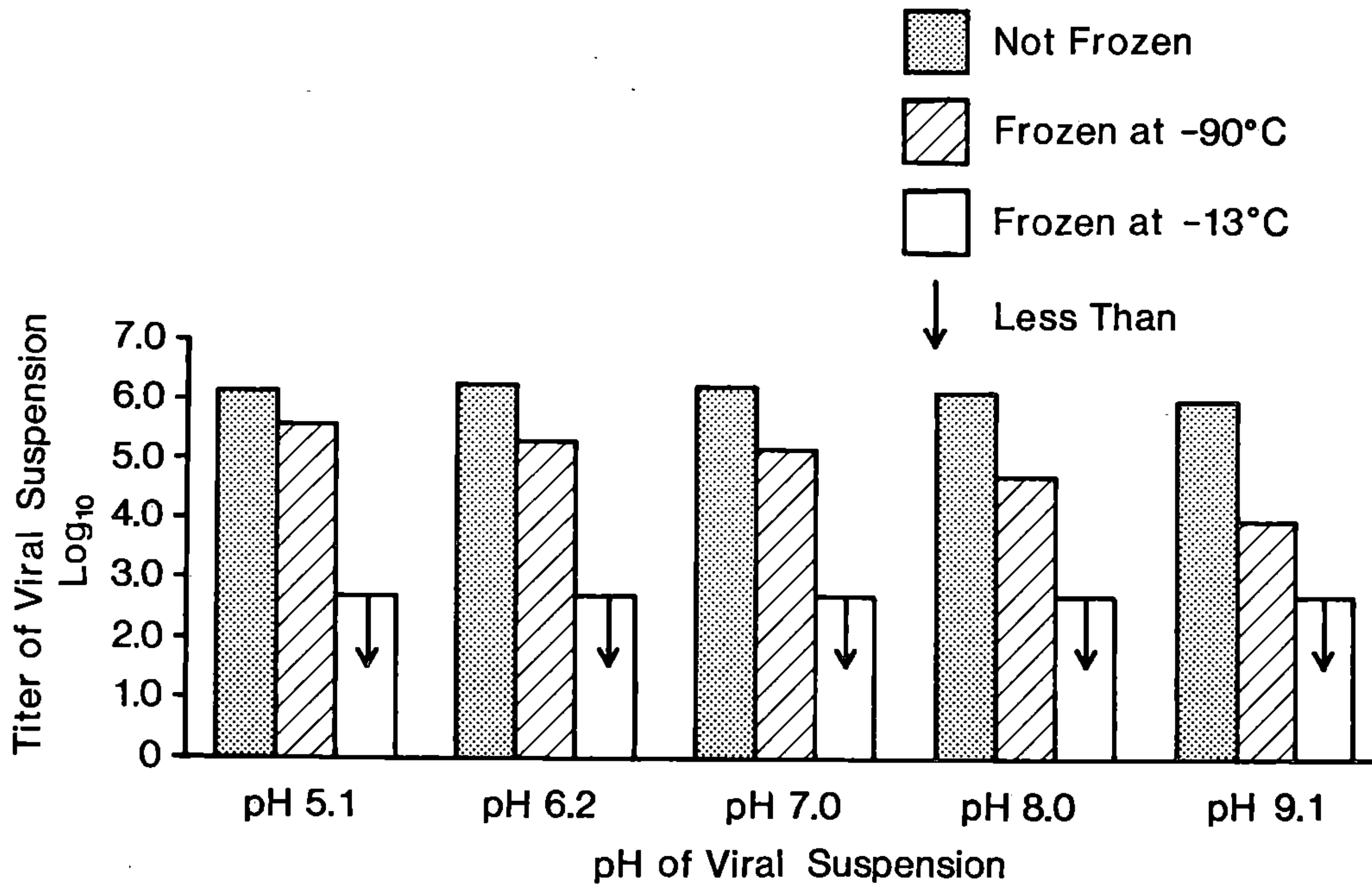
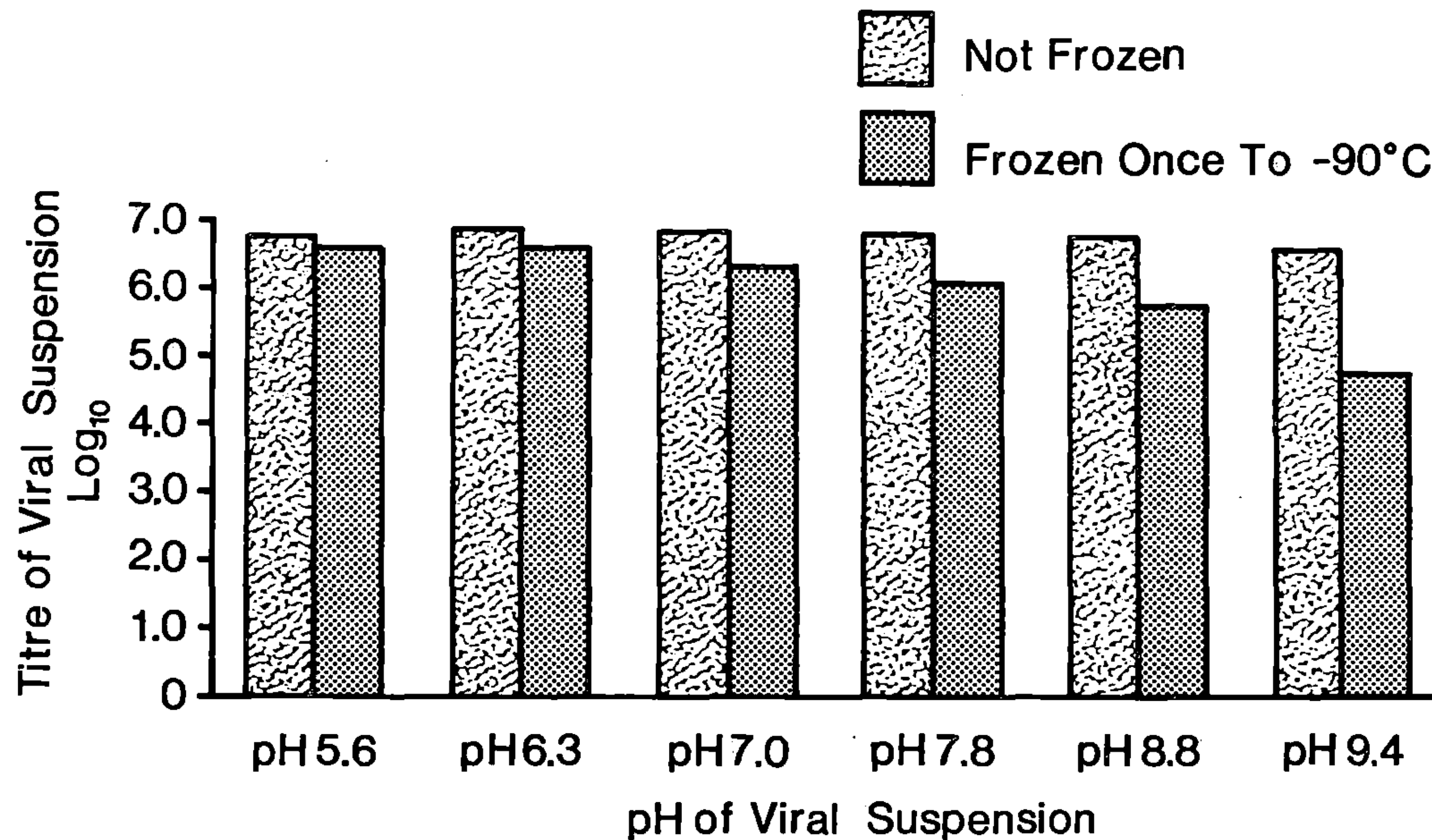
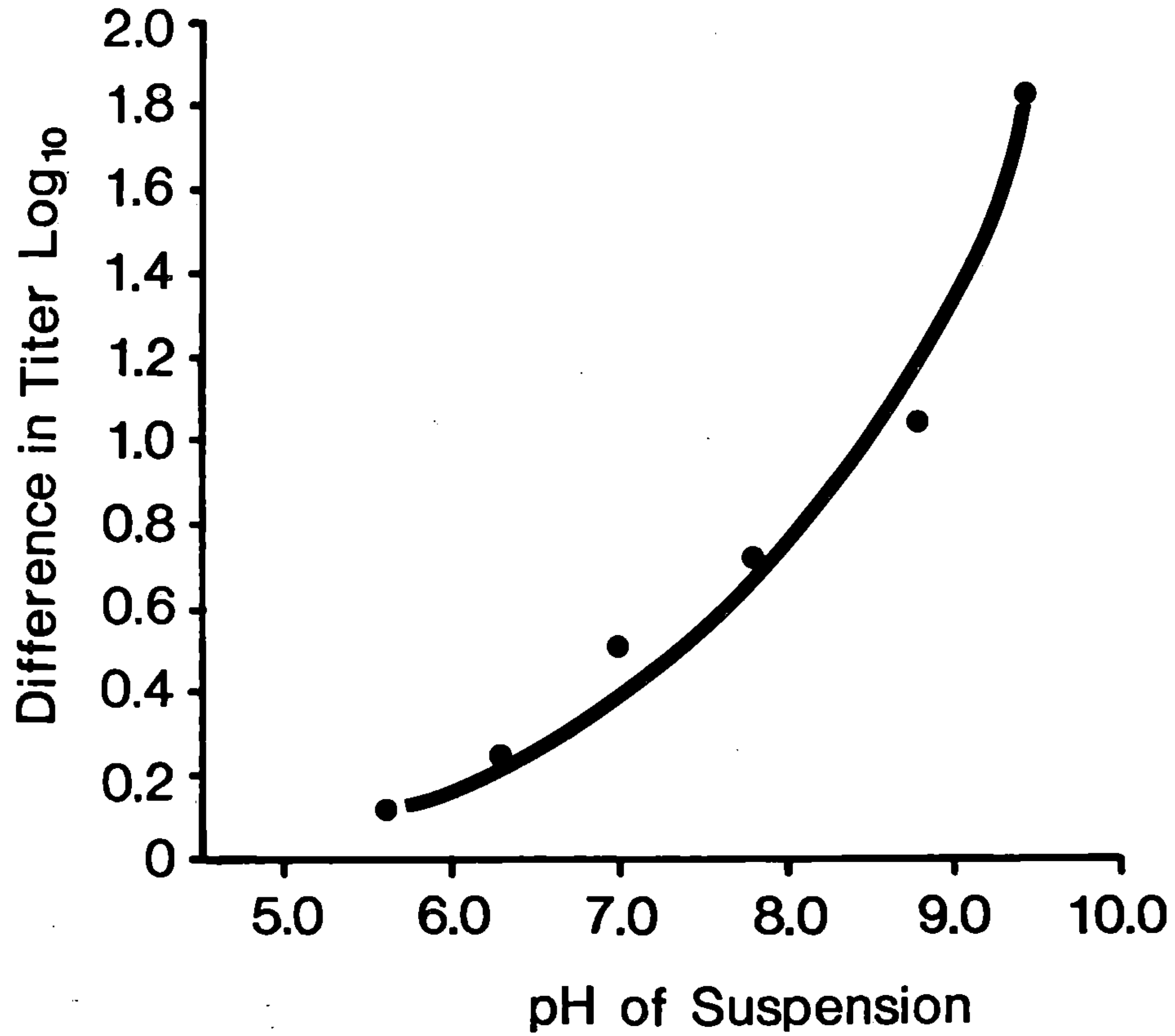

Not Frozen
Frozen at -90°C
Frozen at -13°C
Less Than
Titer of Viral Suspension Log10
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
pH 5.1
pH 6.2
pH 7.0
pH 8.0
pH 9.1
pH of Viral Suspension

Figure 14. The effect of a single rapid freezing to -90°C, compared with the effect of maintenance at 4°C, upon the titer of Aujeszky's Disease virus suspensions at various pH levels



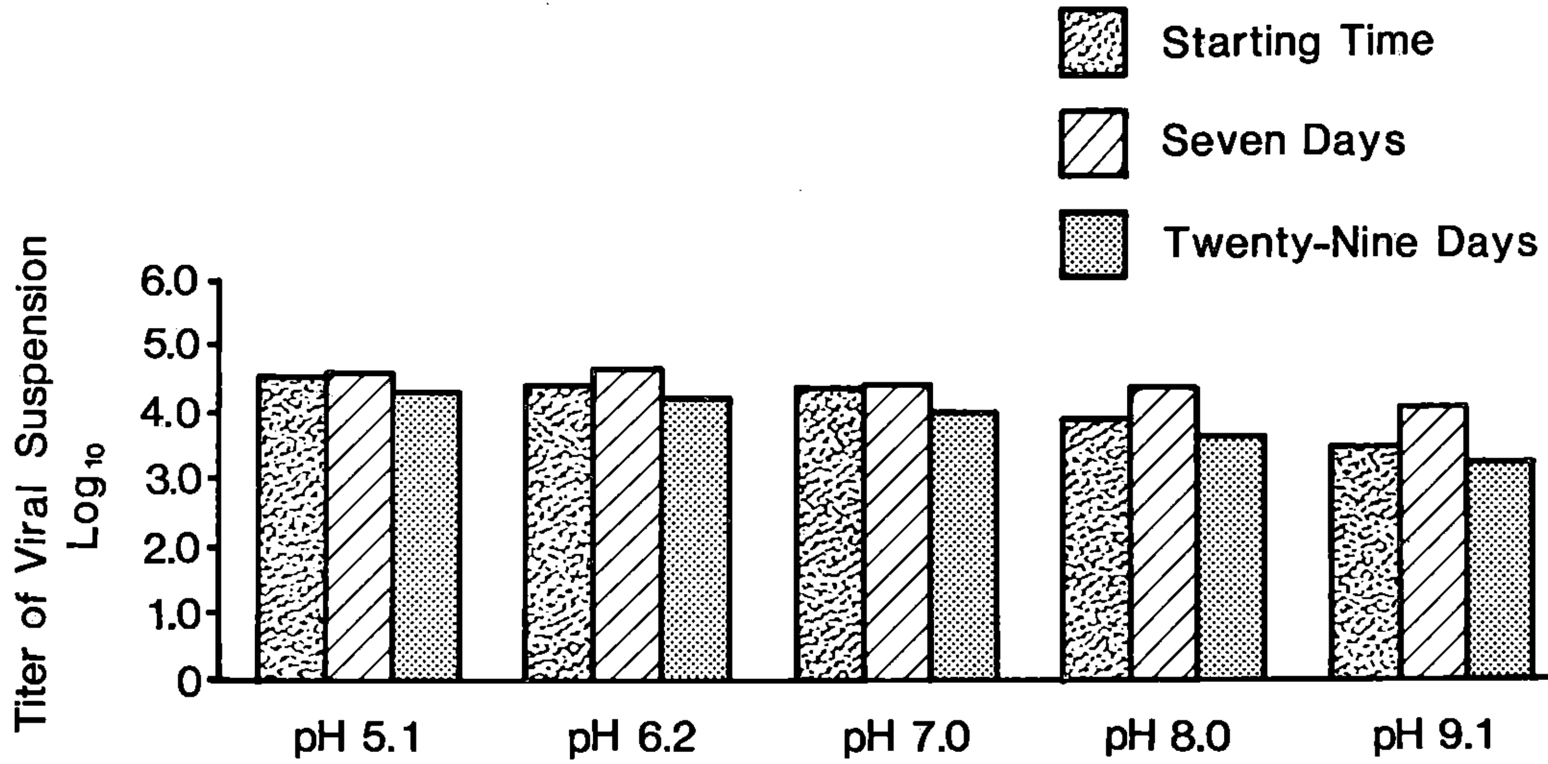
Not Frozen
Frozen Once To -90°C
Titre of Viral Suspension
Log10
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
pH 5.6
pH 6.3
pH 7.0
pH 7.8
pH 8.8
pH 9.4
pH of Viral Suspension

Figure 15. The difference between the titers of Aujeszky's Disease virus suspensions rapidly frozen once to -90°C and held at a constant temperature of 4°C, at various pH levels



Difference in Titer Log10
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
5.0
6.0
7.0
8.0
9.0
10.0
pH of Suspension

Figure 16. The effect of storage at -90°C for a period up to 29 days upon the titer of Aujeszky's Disease virus suspension at various pH levels


Starting Time
Seven Days
Twenty-Nine Days
Titer of Viral Suspension
Log10
6.0
5.0
4.0
3.0
2.0
1.0
0
pH 5.1
pH 6.2
pH 7.0
pH 8.0
pH 9.1

The Effect of Drying and pH Level Upon the Survival of Virus

The fourth group of experiments in this series investigated the effect of drying at various temperatures upon the titer of virus suspensions at various pH levels. Figure 17 shows the effect of drying at $14^{o}C$, $22^{o}C$ and $37^{o}C$ using glass as the substrate, and Figure 18 shows the effect of drying at $37^{o}C$ using gelatin as the substrate.

The Effect of Ultraviolet Light and pH Level Upon the Survival of Virus

The last experiment in this series investigated the effect of exposure to ultraviolet light for time periods of 20, 40 and 60 minutes upon virus suspensions at pH levels ranging from 5.3 to 8.6, as shown in Figure 19. The titers of virus suspensions exposed to the atmosphere but not to ultraviolet light in open Petri dishes on the bench, and virus suspensions held in the vicinity of an ultraviolet light but not exposed to its direct rays are also shown, together with the titers of virus suspensions kept throughout the period of the experiment in sealed tubes in an ice bath.

Figure 17. The effect of drying at temperatures of 14°C, 22°C, and 37°C, upon the titer of Aujeszky's Disease virus suspensions supported on glass, at various pH levels

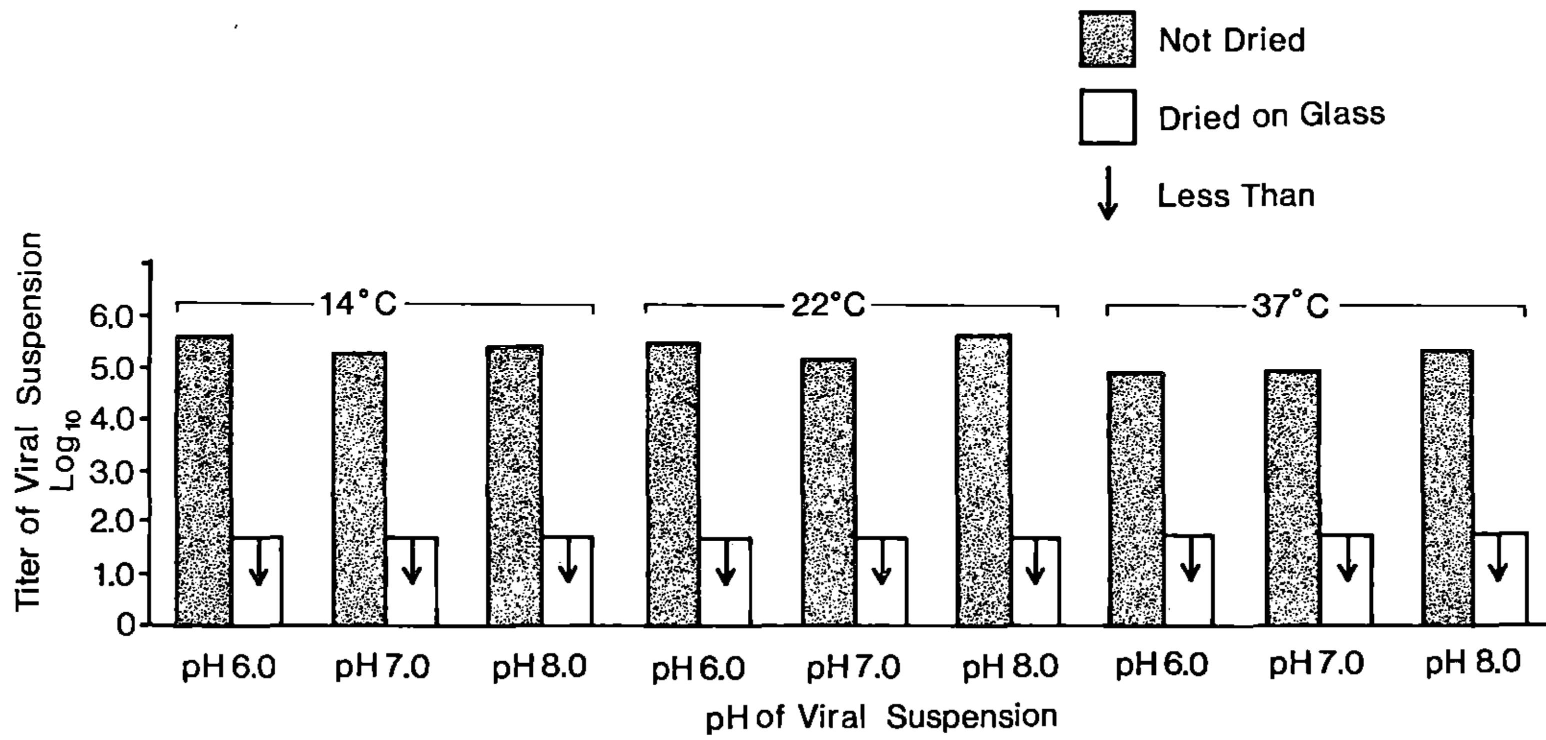

Not Dried
Dried on Glass
Less Than
Titer of Viral Suspension
Log10
6.0
5.0
4.0
3.0
2.0
1.0
0
14°C
22°C
37°C
pH 6.0
pH 7.0
pH 8.0
pH 6.0
pH 7.0
pH 8.0
pH 6.0
pH 7.0
pH 8.0
pH of Viral Suspension

Figure 18. The effect of drying at a temperature of 37°C upon the titer of Aujeszky's Disease virus suspensions supported on gelatin, at various pH levels

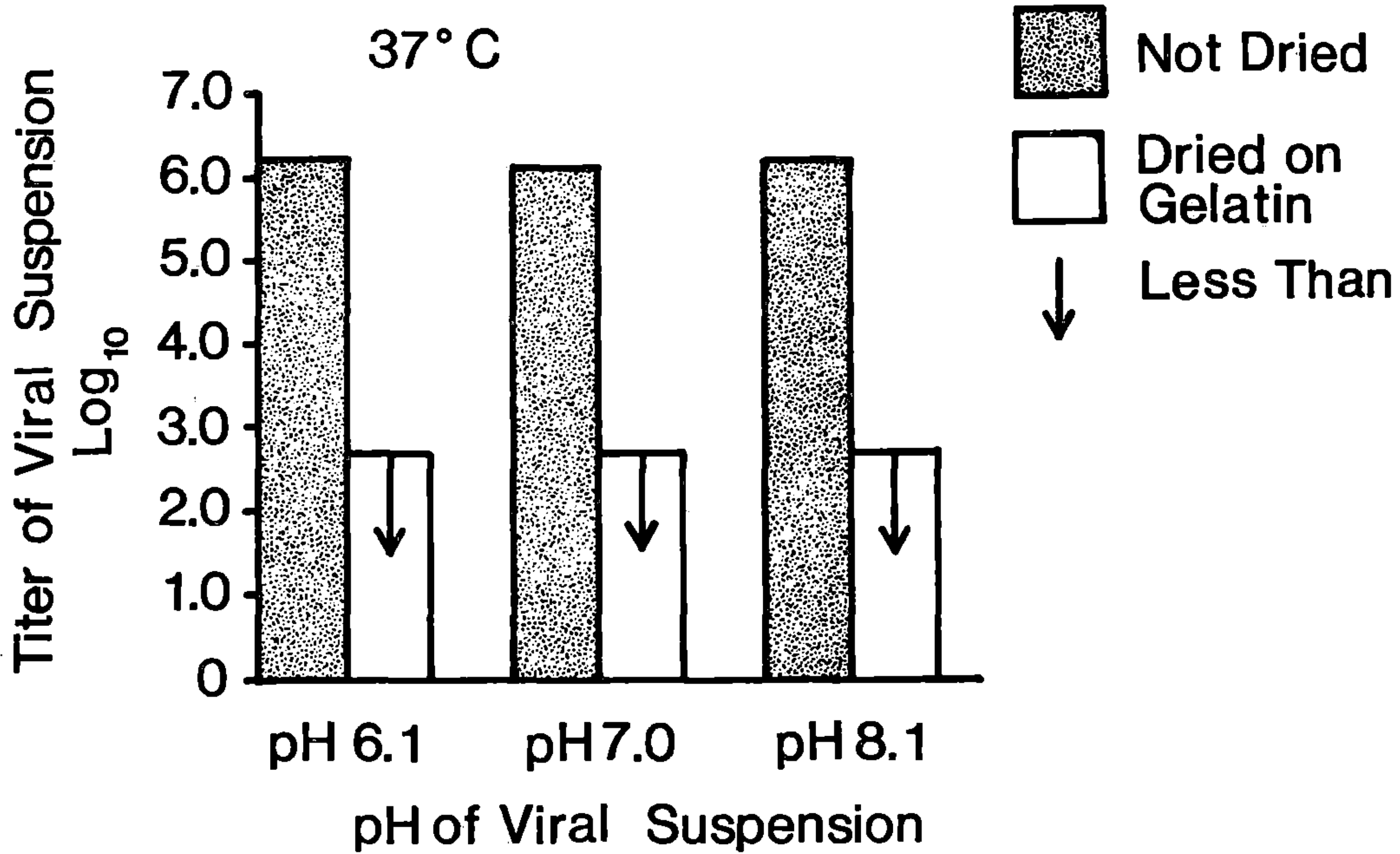
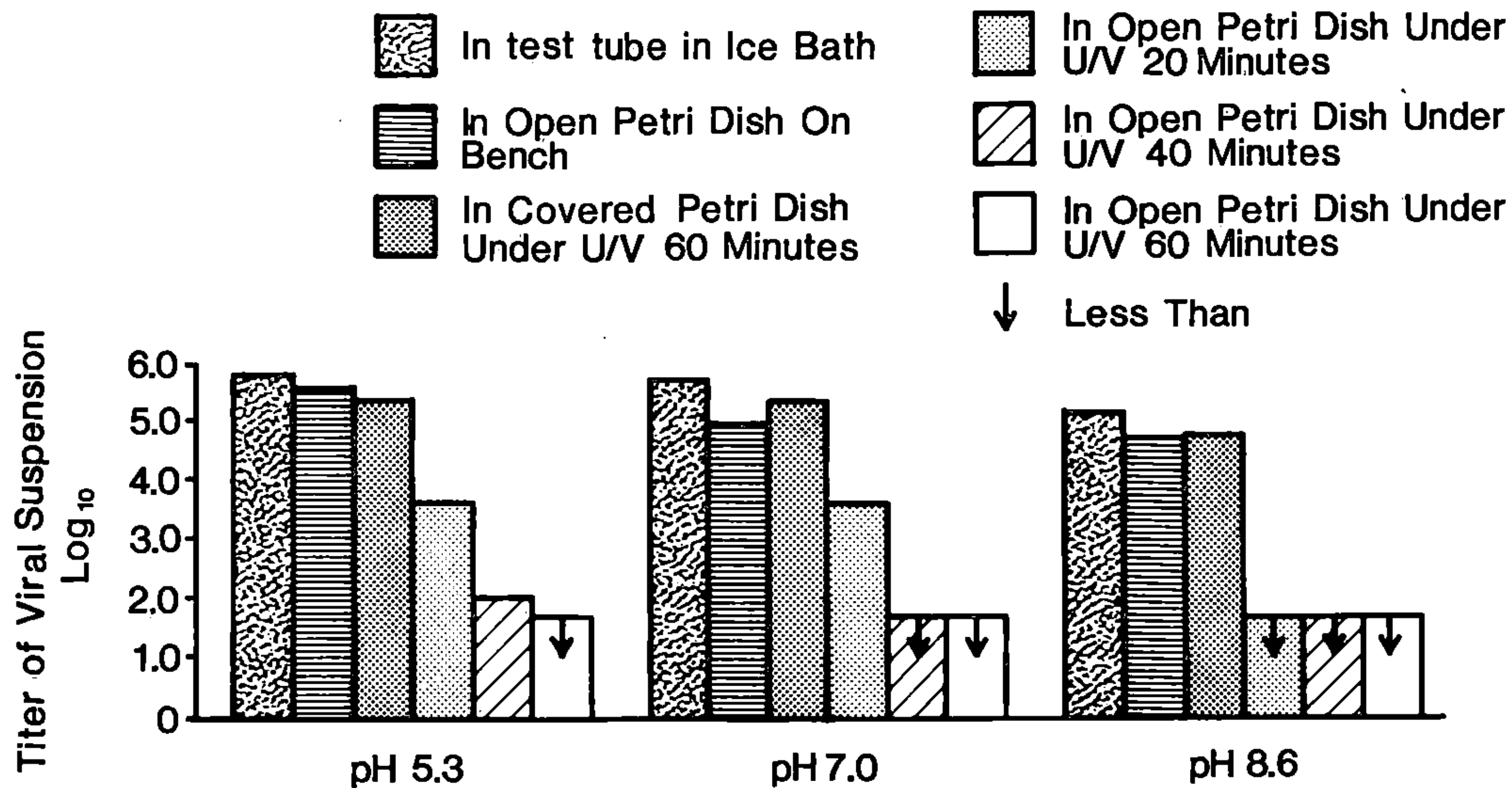

37° C
Titer of Viral Suspension Log10
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
pH 6.1
pH 7.0
pH 8.1
pH of Viral Suspension
Not Dried
Dried on Gelatin
Less Than

Figure 19. The effect of exposure to ultraviolet light for periods of 20, 40 and 60 minutes upon the titer of Aujeszky's Disease virus suspensions held at various pH levels, compared with the effect upon the titer of control suspensions

The controls comprised virus suspensions maintained in closed tubes in an ice bath, in open Petri dishes at ambient temperature but not exposed in any way to ultraviolet light, and in covered Petri dishes near an ultraviolet light and thereby exposed to any atmospheric effect of the light, but not to the direct rays.



In test tube in Ice Bath
In Open Petri Dish On Bench
In Covered Petri Dish Under U/V 60 Minutes
In Open Petri Dish Under U/V 20 Minutes
In Open Petri Dish Under U/V 40 Minutes
In Open Petri Dish Under U/V 60 Minutes
Less Than
Titer of Viral Suspension Log10
6.0
5.0
4.0
3.0
2.0
1.0
0
pH 5.3
pH 7.0
pH 8.6

DISCUSSION

Estimation of the Titer of Virus in a Suspension

Throughout the whole of this series of experiments the amount of virus in a suspension was estimated by using the plaque-counting technique. This system had the advantage of using a highly homogenous population of cells which were automatically randomized during the pooling and centrifugation which was a necessary part of the preparation of the cell culture plates, and so therefore the plates in a particular batch were likely to be very similar in all respects. In addition, before use, the individual plates were randomized and care was taken to ensure that the inoculation procedure was the same in all cases, particularly the period of time of adsorption of virus-containing solution onto the cells. All the plates were held in a single incubator so that they shared a common ambient temperature and atmosphere.

The particular strain of virus which was used in these experiments, produced on these cells clear, easily counted plaques. In general the plaques were counted in those wells which contained as close to 200 plaques each as possible; but in certain survival experiments, as the titer declined it was sometimes necessary to count whatever lesser number was available. It was a standard procedure to inoculate two wells with each dilution of virus-containing solution and to take the average of the resulting plaque counts as the result. Besides giving a better estimate of the true reading, this served to protect to some degree against gross experimental errors, as did the practice of examining

plates as a whole before counting to ensure that the plaque counts progressed in logical tenfold steps along the wells.

As the plaque-counting technique measured what was probably in most cases single virus particles, possibly interspersed with occasional small clumps of virus, and as a total of perhaps 400 plaques were counted in any particular titration, this technique was considered to have yielded more accurate end points than techniques measuring fifty percent lethal dose end points in cell culture tubes or in experimental animals. The plaque technique produced regular replicable graphs at a reasonable cost in materials and labor, and was highly applicable to this study.

Certain Aspects of Experimental Design

During survival studies, it was in many cases necessary to hold samples of virus suspensions for increasing time intervals, and the length of the experiment was therefore necessarily different for different experimental units. This posed a problem in ensuring that all units were identical at the start of the experiment and that they were all measured in an identical manner at the end of the experiment. Three experimental procedures were considered. In the first, the survival of virus at particular temperatures, pH levels and time intervals could be assayed on a common pool of virus prepared to ensure homogeniety at time zero and then divided into the requisite number of samples and adjusted to the required pH levels, an easy and accurate procedure. Aliquots could then be harvested in sequence at the prescribed time intervals and the virus in them measured at that time. Herein lay certain difficulties. Attempts

to titrate the virus at the time of harvest in a large experiment created enormous practical difficulties in preparing suitable cell culture plates all ready at the appropriate times. More importantly, the results as read by plaque counts may not have been comparable from time interval to time interval because the sensitivity of batches of plates could not be assumed to be the same from day to day. The second alternative procedure involved a similar initial phase but instead of titrating each harvest at the time of collection, it would be preserved by freezing and held in this state until all the samples had been collected, when all the samples would be titrated simultaneously in a uniformly prepared batch of cell culture plates. This procedure would be very convenient but, as will be discussed later, freezing had a deleterious effect upon the virus titer and this effect was not constant from one pH to another, as frozen aliquots lost a varying amount of virus during storage with resulting loss of reliability of experimental results.

To counter these difficulties, a different procedure was adopted in which a large number of aliquots of virus were prepared from a common pool and stored together at -90°C. They were expected to all have very similar titers initially and they were expected to maintain these titers for a period sufficient to allow them to be considered identical during the relatively short course of an experiment. Samples were then thawed in turn, their pH adjusted to desired experimental levels, and placed under the appropriate experimental conditions. The sample which was to be tested over the longest period was set up first, the others following in sequence until the "zero time" sample was set up only momentarily on

the day when all specimens were harvested simultaneously and titrated without freezing in one uniform batch of cell culture plates. This procedure was used routinely in experiments involving harvesting at varying time intervals and it worked well. Evidence to support the contention that the titer of aliquots frozen at -90°C may be considered stable for the period of the experiment is given later.

Survival of Virus at Constant Temperatures Above the Freezing Point

One of the most important questions which may be asked of a virus is "how long will it survive in the environment?" Without knowing this, one is not able to give meaningful disease prevention advice to the livestock industry. Fenner et al. (1974) and Fenner and White (1976) considered the majority of pathogenic viruses to be notoriously heat-labile. It has also been shown that the stability of viruses is influenced by the pH of the solutions in which they are contained. To evaluate these two factors, tests were made of the survival of the virus at four constant temperatures above the freezing point (37°C, 25°C, 17°C, and 4°C) each at a range of pH from 4.3 to 9.7. See Figures 1, 3, 5, and 7. As was to be expected on the basis of other viral survival studies, the virus titer showed a steady straight-line decline within the range of titers measured in these experiments when plotted in logarithms against time. Inactivation was more rapid at higher temperatures and there was optimal survival in the middle range of pH, the virus surviving less well at the extremes. The slope of a best-fitting line drawn through points related to a single pH and temperature gave an estimation of the

survival potential of the virus under those particular conditions.

It will be noted that in Figure 1 the titer of the "zero time" sample at pH 4.3 is well-below that of its comparisons at the other pH levels. This is presumably because while in theory nothing can have an effect upon a titer in "zero time", in practice "zero time" amounted to about 15 seconds, and this appeared to have been sufficient time to allow a noticeable loss of virus at this particular temperature-pH combination. It should also be noted that apart from this isolated reading, the rest of the "zero time" titers in the four figures showed good uniformity even though they represented four experiments separated in time by a period of some weeks. This supported the earlier contention that aliquots of a virus solution stored at -90°C maintained their titer for a period sufficient to allow them to be considered identical when preparing serial samples within the course of a relatively short experiment.

The data displayed in Figures 1, 3, 5, and 7 is displayed in another form in Figures 2, 4, 6, and 8 to emphasize the optimum survival of the virus in the pH range of 6.0 to 8.0, and its increasing fragility at pH levels away from this zone. Zůffa and Škoda (1962) spoke of the survival range as being pH 5.0-9.0, a general finding in accord with the more specific determinations presented here. It should be noted that optimal survival occurs in approximately the same pH range for all the temperatures tested.

In each of the figures, the minimum distance between the curve and the abscissa gave a measure of the minimum possible loss in virus titer which might be expected at that temperature. It was found (Figure 2),

that at 37°C and at optimum pH, the titer declined by 0.6 $\log_{10}$ a day. While this was a quite noticeable loss it was still rather less than would have been expected if this virus followed the general statement of Fenner et al. (1974) that the half-life of viruses (i.e., loss of one $\log_2$) at 37°C is measured in minutes. For Aujeszky's Disease virus, this means that at 37°C, an environmental temperature as hot as is likely to be encountered in nature, and taking excretion of infected material from a diseased animal at a titer approaching $10^{7.0}$, then there could still be viable virions in the environment at the end of a 10-day period, assuming no other agent to be either increasing or decreasing the viability of the virus. At lower temperatures, the survival time would be expected to be longer, and viable virus could be expected to remain in infected material of similar titer to that described for 40 days at 25°C, for 60 days at 15°C, and for 120 days at 4°C. In summary, while the Aujeszky's Disease virus is not bizarrely resistant like that of scrapie or kuru, it is nevertheless quite sturdy and well-able to live long enough to be transported from farm to farm on fomites at summer temperatures or to survive over a winter period at temperatures a little above freezing. Figure 9 shows the data from Figures 2, 4, 6, and 8 replotted to demonstrate the relationship of optimal survival at appropriate pH levels to temperature.

While many environmental materials and fomites were found in experiments not described here to occur in the pH zone which was optimal for survival, some did not. Pig saliva for example was normally at a pH of 8.4 to 8.6. While this was not ideal for the virus it was not so far

removed as to have constituted a serious hazard to its survival, and it must be remembered that contact with other material may very well change it. Of great practical importance was the extreme fragility of the virus under acid conditions. Acidification would appear from these experiments to be an effective means of destroying the virus, an observation which supports the work on disinfection by Polyakov and Andryunin (1974) and which was used in this study for the disinfection of glass pipettes.

The Effect of Fluctuating Temperature Upon the Survival of Virus

While constant temperatures were readily maintained in the laboratory, they would be the exception rather than the rule in nature, and it was important that the stability of the virus when subjected to varying temperatures be compared to that at a constant temperature.

In the first experiment to consider this the virus was subjected to a temperature fluctuation between 6°C and 37°C on ten occasions in a period of a little over one hour; the results are illustrated in Figure 10. There was no distinctive change in any of the titers at any of the chosen pH levels and it was therefore concluded that in the normal range of above freezing point temperatures found in nature the virus would not be adversely affected by fluctuations of temperature.

In the second experiment in this series, viral suspensions at selected pH levels were alternately frozen and thawed by alternate immersion of vials in precooled acetone at -90°C and in an ice bath at 4°C. Samples of virus which had not been frozen were titrated at the beginning

of the experiment to establish the starting titers and again at the end of the experiment to ensure that any fall in titer of the thrice-frozen specimens was not due simply to aging on the bench. As is shown in Figure 11, the virus held at ice-bath temperature showed terminal titers indistinguishable from the starting titers, while the virus which was repeatedly frozen and thawed showed progressive loss of titer, particularly at pH 7.9 and 9.0. At this latter pH, the three freeze-thaw cycles produced the quite considerable loss in titer of 3.0 $\log_{10}$.

Continuing this study, the effect of temperature fluctuations below the freezing point was then considered and was tested by raising the temperature of frozen aliquots of various pH levels from $-90^{\circ}C$ to $-13^{\circ}C$ and again lowering them to $-90^{\circ}C$ on 3 occasions over a period of 4 days, the aliquots being allowed to equilibrate at each temperature for a period of approximately half a day. The results of these tests are shown in Figure 12; it will be noted that at no pH level was it possible to detect virus in any of the aliquots which had been raised to $-13^{\circ}C$ and re-lowered to $-90^{\circ}C$. The loss in titer in each case was thus in excess of three $\log_{10}$, which considering the shortness of the experiment and the fact that the virus solution had not been subjected to what would be viewed as harsh treatment, was quite dramatic.

Considering these last two experiments together, it may be asked whether the loss in titer was due to fluctuation of temperature, or whether it was due to the virus entering and being held in a temperature zone which was unfavorable to its survival. In an attempt to shed light on this question, samples of virus of various pH were divided into

aliquots and held at constant temperatures of -90°C and -13°C respectively for 4 days before thawing and measuring for titer. The results of this experiment are shown in Figure 13. It will be noted that the titers of virus titrated without freezing at the time the test samples were put in their respective freezers are also displayed. These served as a check that the titers of the virus at the various pH levels were uniform at the start of the experiment, and gave some indication of the drop in titer during freezing, but care should be taken not to read too much into the individual comparisons of frozen and unfrozen material, because as stated the respective titrations were performed on different days with perforce different batches of cell culture plates, and the sensitivity of the cell cultures may not have been constant. It was however, legitimate to note the stepwise decline with rising pH of the titer of the virus held at -90°C and to conclude that virus to be stored at this temperature should be adjusted to a pH between 6.0 and 7.0 to minimize loss of titer. Virus which was stored at -13°C for four days lost all detectable viability, a loss of at least three $\log_{10}$ in titer.

To summarize the conclusions which may be drawn from these experiments with fluctuating below zero temperature, it would appear that the virus was highly unstable at temperatures a little below freezing, as suggested by Žuffa and Škoda (1962) and Maré (1975). Whether the fluctuation of the temperature had any particular effect upon the virus in addition to the deleterious effect of the adverse temperature zone at which it was held could not be determined from this series of experiments. It can only be noted that from a practical point of view, under conditions

of field outbreaks of disease in wintertime the enhanced fragility of the virus at temperatures just below freezing could be welcomed and may well be very important epidemiologically.

Long-Term Storage of the Virus in the Laboratory

While the enhanced fragility of the virus at just subzero temperatures may be an asset during epidemics of Aujeszky's Disease in the field in wintertime, it is a factor which must be considered in storage of the virus in the laboratory. In laboratory storage it is desired to put the virus suspension into the frozen state, to hold it for perhaps years in the frozen state, and to return it to the liquid state, with minimum loss of titer.

So as to determine the loss of titer caused by a very rapid freeze-thaw cycle passing through the destructive subzero zone as quickly as possible, vials of virus suspensions at various pH levels were immersed in precooled acetone at -90°C for sufficient time for them to freeze and for their temperature to equilibrate; they were then rapidly thawed in an ice bath at 4°C. The whole freeze-thaw cycle took approximately half an hour. The solutions were then titrated together with controls which had been maintained for this period at 4°C in cell culture plates prepared from a common pool of cells; the results are shown in Figure 14. It will be noted that, as expected (Figure 7), there was little discernable difference in the titers of the unfrozen virus suspensions at the various pH levels. It will also be noted that there was at each pH a loss of titer associated with the freeze-thaw cycle, and that while this loss

was minimal at pH 5.6 it increased sharply with rising pH. These data have been transformed to emphasize this point in Figure 15. Clearly to avoid loss of titer of virus while freezing the pH should be adjusted before starting so that it does not exceed 7.0.

Besides considering the effect of quick freezing and thawing, the effect of low temperatures on the long-term storage of virus was also assayed. This has already been discussed to some extent earlier. The consistency of the "zero-time" titers in the series of experiments Figures 1, 3, 5, and 7 indicated that stock virus held at -90°C maintained its titer for some weeks at least. It was also noted in experiments which are not described here that this virus maintained its titer for months during storage at -90°C. It should however, be noted that the standard stock virus solution used in all these experiments was at pH 6.8, and so to ascertain the effect, if any, of pH level upon virus during storage at -90°C a further experiment was conducted as shown in Figure 16. It was seen that the virus survived well for 29 days at all pH levels ranging from 5.1 to 9.1. All the titers at the two higher pH levels were marginally lower than the other titers, possibly because of a greater deleterious effect of the freezing and thawing procedures.

The Effect of Drying Upon the Virus

While virus kept in the laboratory in sealed tubes did not suffer the hazard of drying, in nature this is a prominent feature of the environment, and it was important to ascertain whether drying had any deleterious effect upon the virus.

The first experiment, illustrated in Figure 17, shows the effect upon the titer of virus at three different pH levels, of drying on a glass surface as compared with similar virus passed through the same temperature cycle but prevented from drying by being in sealed tubes. No virus could be detected after drying, the loss in titer being in excess of 3 $\log_{10}$.

It may however, be asked if the virus had in fact been inactivated, or if it had simply adhered to the glass and was not released during the subsequent rehydration. If this was so it could be of practical importance in field conditions if virus behaved similarly on an edible material. While it may not be released by elution, it might be released in the digestive tract of a susceptible animal. Accordingly, a second experiment was conducted, in which the virus was this time dried on gelatin, a substrate which was subsequently dissolved. The results are shown in Figure 18, and it will again be seen that no virus was recovered, it having again suffered a loss in titer in excess of 3 $\log_{10}$. The presence of dissolved gelatin in the (undried) control did not affect its titer, so the virus cannot be said to be adsorbed and retained on gelatin molecules.

It does therefore appear that drying had a most serious effect upon the virus when suspended in Saline G. It may be that the progressive concentration of electrolytes in the solution as evaporation occurred exerted the detrimental effect, rather than the actual drying. If so, an electrolyte-free medium might better sustain the virus. Clearly there is room here for further study.

The Effect of Ultraviolet Light Upon the Virus

It is well-known that U/V light has a detrimental effect upon many microorganisms. This is of great practical importance in both the epidemiology of the disease in the field and in the sterilization of laboratory working environments. This series of experiments was concerned only with the behavior of the virus in the laboratory, and tests were restricted to artificial U/V light produced by a commercial U/V bulb described by the manufacturer as being "germicidal". The virus was exposed to it in a situation of temperature and distance from the bulb designed to simulate an accidental bench-top spill in the presence of such a light. The results of this experiment are given in Figure 19. It will be seen that virus held the whole period in sealed tubes at $4^{o}C$ to give a measure of the starting titer showed approximately uniform results, albeit possibly a fraction lower at the higher pH. Virus which was held in open Petri dishes on the bench at the ambient temperature but not in any way exposed to the effects of U/V light showed a marginal presumably temperature related loss in titer. Virus held in Petri dishes covered with aluminum foil under the U/V light for 60 minutes, thereby being exposed to any change in atmosphere such as to ozone produced by the U/V light but not to the light itself, was little different in titer. The titer of the virus which was exposed to the direct rays of the light however showed a marked decline, particularly at pH 8.6, but even at the other pH levels the virus suffered a loss of over 3 $\log_{10}$ in 40 minutes. It was apparent that the manufacturer's claim that their lamp was germicidal was well-founded as regards Aujeszky's Disease virus and it would appear

to be appropriate technique to use such lamps in laboratory hoods and working areas whenever possible, subject to the restriction that the lamps cannot be used in the direct presence of personnel because of their adverse effects upon the eyes. It is also to be presumed from this experiment that in field conditions ultraviolet light from the sun may well shorten the survival period in virus in exposed environments and so be of epidemiological importance.

Limitations of This Work

In this work a wide range of factors which influence the survival of Aujeszky's Disease virus have been assayed in the laboratory. It is hoped that the findings will be of interest to those working in the control of Aujeszky's Disease as well as to those handling the virus in the laboratory.

It is however, proper that the limitations of any piece of work should be pointed out to avoid attributing to it a wider implication than it deserves. The limitations of the present work were twofold. Firstly, it dealt with only one strain of Aujeszky's Disease virus. This strain was however, deliberately chosen as a representative recent field strain found in Iowa, and was tested using as few laboratory passages as was practical. It is therefore hoped that it is similar to many field strains.

The second limitation to this work is that it was conducted throughout using Saline G - an isotonic buffered saline containing protein - as the suspending medium. This was chosen as the medium because

it was expected to simulate the typical body fluids or secretions of an animal. In real life there is an infinite variety of solutions in which a virion may find itself. It is hoped that the choice in this case has been appropriate and that the results have useful applications.

SUMMARY

The ability of Aujeszky's Disease (pseudorabies) virus to survive outside the living host at constant temperatures above the freezing point was found to be strongly correlated with pH level and temperature. The optimum pH for survival was found to be between 6.0 and 8.0. Rapid inactivation occurred at pH 4.3. At optimum pH level for survival, virus inactivation occurred at a rate varying from 0.04 $\log_{10}$/day at 4°C to 0.6 $\log_{10}$/day at 37°C.

Fluctuation of the temperature between 4°C and 37°C had no apparent effect upon the titer of the virus.

At a steady temperature just below freezing (-13°C), the virus was rapidly inactivated at all tested pH levels.

For long-term storage it is recommended that the pH of Aujeszky's Disease virus suspensions should be adjusted between 6.0 and 7.0, that they should be kept at -90°C, and that freezing and thawing should be rapid so as to pass through the just subzero temperature zone as quickly as possible.

Drying, both on glass and on gelatin, caused inactivation of the virus at all pH levels, as did exposure to ultraviolet light.

BIBLIOGRAPHY

Akkermans, J. P. W. M. 1974. Aujeszky's disease and meat inspection of cattle (in German, English summary). Tijdschr. Diergeneeskd 99 (11):577-578.

Atanasova, Kh. 1972. Resistance of Aujeszky's disease virus in the urine of pigs and sheep (In Bulgarian, English, Russian). Veterinarnomeditsinski Nauki, Sofia 9(9):69-74. Abstracted by the Veterinary Bulletin 43:1066. 1973.

Aujeszky, A. 1902. A report of a new infectious disease of domestic animals (in German). Z. Bakteriol. Abt. I. Orig. 32:353-357.

Baskerville, A., J. B. McFerran, and C. Dow. 1973. Aujeszky's disease in pigs. Veterinary Bulletin 43:465-480.

Beran, G. W., A. V. Zierke, H. T. Hill, and C. J. Maré. 1976. An epidemiological study of pseudorabies in a rural Iowa community with emphasis on its economic impact. Proc. Internat. Pig Vet. Soc. (1976 Congress, Ames, Iowa), p. G.4.

Braga, A. and A. Faria. 1934. Infectious bulbar paralysis (in Spanish). Rev. Dept. Nac. Produccao Animal (Rio de Janiero) 1:53-138. (English Resumé 110-115.

Brierer, B. W. 1939. History of animal plagues of North America. Reprinted by United States Department of Agriculture, Washington, D.C. 1974. 97 pp.

Cassells, R. W., and H. G. Lamont. 1942. Aujeszky's disease in dogs. Vet. Rec. 54:21-23.

Csontos, L., L. Héji, and I. Szabó. 1962. A contribution to the Aetiology of Aujeszky's disease in the pig - Foetal damage and abortion due to the virus. Acta Vet. Acad. Sci. Hung. 12:17-23.

Cultivator (Albany). Vol. 6. 1839-40. Page 182. Excerpt reprinted. Page 12 _in_ History of Animal Plagues of North America. Brierer, B. W. 1939. Reprinted by United States Department of Agriculture, Washington, D.C. 1974.

Dillman, R. C., and J. J. Andrews. 1975. Pseudorabies in Iowa swine: An increasing problem. Proc. Am. Assoc. Vet. Lab. Diagnosticians 17:145-155.

Fenner, F., and D. O. White. 1976. Medical virology. 2nd edition. Academic Press, New York. 487 pp.

Fenner, F., B. R. McAuslan, C. A. Mims, J. Sambrook, and D. O. White. 1974. The biology of animal viruses. Academic Press, New York, 834 pp.

Galloway, I. A. 1938. Aujeszky's disease. Vet. Rec. 50:745-762.

Glukhov, V. F. 1971. Role of ticks in the transmission of Aujeszky's disease (in Russian). Veterinariya, Moscow 7:46-48. Abstracted by the Veterinary Bulletin 42:3798. 1972.

Golais, F., and A. Sabó. 1975. The effect of temperature and urea on virulent and attenuated strains of pseudorabies virus. Acta Virol. 19:387-392.

Gordon, W. A. M., and D. Luke. 1955. An outbreak of Aujeszky's disease in swine with heavy mortality in piglets, illness in sows and deaths in utero. Vet. Rec. 67:591-597.

Gore, R., A. D. Osborne, P. G. G. Darke, and J. N. Todd. 1977. Aujeszky's disease in a pack of hounds. Vet. Rec. 101:93-95.

Gustafson, D. P. 1975. Pseudorabies. Pages 391-410 _in_ H. W. Dunne ed. Disease of swine. 4th edition. The Iowa State University Press, Ames, Iowa. 1212 pp.

Hill, H. T., and C. J. Maré. 1975. Diagnostic features of recent pseudorabies (Aujeszky's disease) outbreaks in central United States. Proc. Am. Assoc. Vet. Lab. Diagnosticians 18:453-458.

Howarth, J. A. 1969. A serological study of pseudorabies in swine. J. Am. Vet. Med. Assoc. 154:1583-89.

Ivanov, I., Kh. Kharalambi, and B. Stamenov. 1968. Investigations into Aujeszky's disease in sheep (in Bulgarian, English and Russian summaries). Vet. Med. Nauki, Sof. 5(4):9-18.

Kanitz, C. L. 1977. The role of wildlife and domestic animals in the epidemiology of pseudorabies. Pseudorabies Fact-Finding Conference, Iowa State University, Ames, Iowa.

Kaplan, A. S. 1969. Herpes simplex and pseudorabies virus - properties of the viruses. Virol. Monogr. 5:7-26.

Karasszon, D. 1965. Pathohistological studies on the central nervous system of monkeys inoculated with a "modified" strain of Aujeszky's disease. Acta Vet. Hung. 15:405-413.

Kluge, J. P., and C. J. Maré. 1974. Swine pseudorabies: Abortion, clinical disease, and lesions in pregnant gilts affected with pseudorabies virus (Aujeszky's disease). Am. J. Vet. Res. 35:911-915.

Kojnok, J. 1957. Mother's milk and the spread of Aujeszky's disease in suckling pigs. Acta Vet. Hung. 7:273-276.

Kretzschmar, C., and W. Schulz. 1964. The spread of Aujeszky's disease in the vicinity of Magneburg (in German). Mh. VetMed 19:293-299.

Ljubashenko, S. J., A. F. Tjul'panova, and V. M. Grishin. 1958. Aujeszky's disease in mink, arctic fox and silver fox (in Russian). Veterinariya, Moscow 35:37-41. Abstracted by the Veterinary Bulletin 29:1386. 1959.

Maré, C. J. 1975. Pseudorabies (Aujeszky's disease): The virus and its pathogenesis in pregnant swine. Symposium on pseudorabies (Aujeszky's disease), Peoria, Illinois. Livestock Conservation Inc., Omaha, Nebraska.

Maré, C. J., J. P. Kluge, and G. W. Beran. 1976. Reproductive failure in swine infected with pseudorabies (Aujeszky's) virus. Proc. Internat. Pig Vet. Soc. (1976 Congress - Ames, Iowa). p. G1.

Mašić, M., and M. Petrović. 1961. Failure to transmit Aujeszky's disease by lice (in Serbian, summary in German). Acta Vet. Yugoslav. 11:79-84. Abstracted by the Veterinary Bulletin 31:3945. 1961.

McCrocklin, S. M. 1976. Studies in the role of wild mammals in the spread of pseudorabies among swine. M.S. Thesis. Purdue University. 90 pp.

McFerran, J. B., and C. Dow. 1964. The excretion of Aujeszky's disease by experimentally-infected pigs. Res. Vet. Sci. 5:405-410.

McFerran, J. B., and C. Dow. 1965. The distribution of the virus of Aujeszky's disease (pseudorabies virus) in experimentally infected swine. Am. J. Vet. Res. 26:631-635.

Nicolau, S., L. Cruveilhier, and L. Kopliowska. 1937. Experimental study of pseudorabies (Aujeszky's disease). Compt. Rend. Soc. Biol. 126:563-565.

Nikitin, M. G. 1961. Pigs as carriers of Aujeszky's disease virus (in Russian). Veterinariya, Moscow 38(9):32-36. Abstracted by the Veterinary Bulletin 32:1087. 1962.

Nikolitsch, M. 1954. Aujeszky's disease in roe deer in Yugoslavia (in German, English, French and Italian summaries). Wien. Tierärztl. Mschr. 41:603-605.

Platt, K. B. 1977. Biological markers for the differentiation of Aujeszky's disease virus strains. Ph.D. Thesis. Iowa State University. 166 pp.

Platt, K. B., and C. J. Maré. 1976. Comparison of the biological characteristics of virulent and attenuated pseudorabies (Aujeszky's) virus. Proc. Internat. Pig Vet. Soc. (1976 Congress - Ames, Iowa). p. G11.

Polyakov, A. A., and Yu. I. Andryunin. 1974. Disinfection of liquid manure contaminated with foot and mouth disease and Aujeszky's disease viruses (in Russian). Veterinariya, Moscow 5:45-47. Abstracted by the Veterinary Bulletin 44:4357. 1974.

Pseudorabies Fact-Finding Conference, Iowa State University, Ames, Iowa. 1977. U.S. Government Printing Office, Washington, D.C. 130 pp.

Remlinger, P., and I. Bailly. 1934. A contribution to the study of the virus of Aujeszky's disease (in French). Ann. Inst. Pasteur 52: 361-405.

Remlinger, P., and I. Bailly. 1939. Transmission of Aujeszky's disease to a turkey (in French). Bull. Acad. Vet. Fr. 12:362-365.

Sabó, A. 1969. Persistance of perorally administered virulent pseudorabies virus in the organism of non-immune and immunised pigs. Acta Virol. 13:269-277.

Sabó, A., and J. Rajčáni. 1976. Latent pseudorabies virus infection in pigs. Acta Virol. 20:208-214.

Saunders, J. R., and D. P. Gustafson. 1964. Serological and experimental studies of pseudorabies in swine. Proc. U.S. Livestock San. A:256-265.

Saunders, J. R., D. P. Gustafson, H. J. Olander, and R. K. Jones. 1963. An unusual outbreak of Aujeszky's disease in swine. Proc. U.S. Livestock San. A:331-346.

Schmiedhoffer, J. 1910. A study of the pathology of infectious bulbar paralysis (Aujeszky's disease) (in German). Z. Infektionskrankh. Parasitenk. Krankh. Hyg. Haustiere 9:383-405.

Schneider, W. J., and T. M. Haslett. 1978. Attempted experimental transmission of pseudorabies virus to European starlings. J. Wildl. Dis. 14:170-172.

Scott, E. M., and W. Woodside. 1976. Stability of pseudorabies virus during freeze-drying and storage: Effect of suspending media. J. Clin. Microbiol. 4:1-5.

Shope, R. E. 1931. An experimental study of "Mad Itch" with especial reference to its relationship to pseudorabies. J. Exp. Med. 54: 233-248.

Shope, R. E. 1932. Identity of the viruses causing "Mad Itch" and pseudorabies. Proc. Soc. Exp. Biol. Med. 30:308-309.

Shope, R. E. 1934. Pseudorabies as a contagious disease in swine. Science 80:102-103.

Shope, R. E. 1935a. Experiments on the epidemiology of pseudorabies - mode of transmission of the disease in swine and their possible role in its spread to cattle. J. Exp. Med. 62:85-99.

Shope, R. E. 1935b. Experiments on the epidemiology of pseudorabies - prevalence of the disease among middle western swine and the possible role of rats in herd-to-herd infections. J. Exp. Med. 62:101-117.

Simeonov, S. 1973. Carriage and excretion of Aujeszky's disease virus by pigs (in Bulgarian, English and Russian summaries). Veterinarno Meditsinski Nauki, Sofia 10(1):67-72.

Solomkin, P. S., and M. I. Tutushin. 1956. Viability of the virus of Aujeszky's disease in fodder and on products of animal origin (in Russian). Veterinariya, Moscow 33(4):49-50. Abstracted by the Veterinary Bulletin 26:3448. 1956.

Stewart, W. C. 1975. Is pseudorabies (Aujeszky's disease) increasing in the United States? Symposium on pseudorabies (Aujeszky's disease), Peoria, Illinois. Livestock Conservation Inc., Omaha Office, 604 Livestock Exchange Bldg., Omaha, Nebraska.

Symposium on Pseudorabies (Aujeszky's Disease). 1975. Peoria, Illinois, Livestock Conservation Inc., Omaha Office, 604 Livestock Exchange Bldg., Omaha, Nebraska. 44 pp.

Trainer, D. O., and L. Karstad. 1963. Experimental pseudorabies in some North American mammals. Zoonoses Res. 2:135-151.

Tuncman, Z. M. 1938. Aujeszky's disease seen in man (in French). Ann. Inst. Pasteur 60:95-98.

Ustenko, V. S. 1957. Survival of the virus of Aujeszky's disease (in Russian). Veterinariya, Moscow 34(3):74-75.

Ustenko, V. S. 1958. Survival of the virus of Aujeszky's disease in the external environment (in Russian). Trudy Vsesoyuz. Inst. Vet. Sanit. i. Ektoparasit. 13:49-59.

Venturoli, F. 1975. Resistance of Aujeszky's disease virus to the action of disinfectants (in Italian, German and English summaries). Anali della Facoltà di Medicina Veterinaria di Torino 22:233-240.

Žuffa, A., and R. Škoda. 1962. Some biological properties of Aujeszky's virus (in Slovak, English, German and Russian summaries). Vet Čas. 11:155-164.

ACKNOWLEDGMENTS

This study would not have been possible without the loyalty and courage of my wife Joan and my son Giles who accepted without complaint my absence in another country for almost two years while the work was completed. They will understand that I cannot adequately express the warmth of my feelings for them in cold print.

I also wish to thank Dr. G. W. Beran, who rather adventurously agreed to be my major professor without having met me and who has since given me inestimable support throughout this project, Dr. R. A. Packer, who again without having met me accepted me into this department and who has since advised me and served on my committee, and Dr. W. A. Rowley, of the Entomology Department, who has besides supporting me by serving on my committee, given breadth to my program.

The work in the laboratory was made pleasurable by the efficient technical assistance of Mrs. Dorothy Murphy, who remained cheerful even in the face of disaster. There were many disasters to test her fortitude. Despite them all she kept smiling.

The preliminary typing of this thesis to convert my illegible script into readable print was done by my friend Mrs. Marge Davis with no thought of reward. I thank her sincerely. Without her help I should have been in serious difficulty.

There is more to be gained from visiting another country to get a degree than just the course of instruction. The contact with the people of that country is much more valuable. I wish to thank the faculty and

students of this whole campus and also the people of Ames for the courteous way in which they have welcomed me into their community and made me feel at home. I shall always have happy memories of the time I spent in the midwest of America.