MODIFICATION OF THE HOG CHOLERA VIRUS

by

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INTRODUCTION

Hog cholera, described by Hagan and Bruner (31) as an acute, highly contagious disease, caused by a filterable virus, has claimed the attention of research workers for many years because of its wide distribution, its great economic importance, and its scientific interest. Salmon (54) reported that the first outbreak of hog cholera in the United States probably occurred in Ohio in 1833 and that it seemed reasonably certain the disease was imported from Europe. The disease is now widespread in the United States. It appears each year, and in some years its effect has been devastating to the swine industry. Mohler, Wight, MacKeller, and Bishopp (48) estimated that, as of January 1, 1942, the annual loss in the United States from hog cholera is twelve and one-half million dollars. Hutyra, Marek, and Manninger (37) state that hog cholera now occurs in almost all European countries, as well as in Africa, Australia, China, and Japan.

Naturally, with a disease of such world-wide economic importance, the majority of investigations dealing with it have been attempts to produce effective means of control and prevention. For many years, control and prevention of hog cholera have been based on the immunization of swine by the simultaneous injection of virulent virus and anti-hog cholera serum. This method is effective, but, since the active, disease-producing agent is employed, foci of infections have been maintained. For this reason, and especially because the immunizing materials have been used by many persons unmindful of the danger associated with the use of a live virus,

hog cholera has remained a major disease problem for the swine industry.

The need for an immunizing agent that affords adequate protection, but is at the same time incapable of spreading the disease, has long been recognized. The development of Boynton Tissue Vaccine (B.T.V.) and crystal-violet vaccine resulted from efforts to meet this need. The virus in these two products was attenuated by means of chemical treatment. Because of certain inherent limitations, these vaccines have not been entirely satisfactory, although the hazards associated with the use of a virulent virus are avoided. Other methods of attenuation studied prior to 1946 had resulted in failure to produce an avirulent, antigenic virus. Certain lines of approach, however, to the problem of producing a satisfactory hog cholera vaccine remained unexplored or only partially investigated: modification or adaptation to an abnormal host, ultraviolet irradiation, and inactivation by other chemicals.

Certain viruses have been adapted to animals other than the so-called normal host. In some instances the adapted virus became modified in pathogenicity for its host but retained its immunizing potency. Baker's (3) successful adaptation, by an alternate passage technique, of the rinderpest virus to rabbits suggested that the hog cholera virus might also be adapted to rabbits. Both viruses are highly host-specific, and, in the case of the hog cholera virus, attempts to infect animals other than the pig failed. The alternate passage technique opened another avenue in endeavors to produce an attenuated live hog cholera virus of great immunizing possibilities.

The inactivation of certain viruses by ultraviolet light has been accomplished by many workers. In some cases the virulence of the virus was destroyed, but the attenuated virus remained antigenic. The effect of ultraviolet irradiation on the hog cholera virus has not been studied, although it offered a possibility of attenuation without loss of immunizing potency. If such treatment resulted in the production of an avirulent virus, the method would be capable of wide-spread application.

The development of certain nitrogen mustards and studies of their effects suggested that these agents might also offer a means of attenuating the hog cholera virus. Tenbroeck and Herriott (57) reported the results of their study of the effect of a mustard (Bis (B-chloroethyl) Sulfide) on several viruses, including the hog cholera virus. They found that 18 of 23 pigs injected with a mustard-treated hog cholera virus showed some immunity. This brief report left many questions unanswered, but it definitely indicated that this line of investigation should be extended.

Since these three methods of studying the hog cholera virus seemed in need of further exploration, the work reported in this thesis consisted of efforts to modify or attenuate the virus by adaptation to the rabbit, by ultraviolet irradiation, and by treatment with nitrogen mustard. The ultimate end in view was, of course, the production of a satisfactory immunizing product without the limitations associated with the use of a virulent virus.

After formulation of plans for this investigation, papers by Baker (4) and Koprowski, James, and Cox (42) appeared on the subject

of passage of the hog cholera virus to rabbits. The findings of these workers differed in several respects, and it became even more important that the question of rabbit adaptation of the virus be further investigated. The influence on the results of the strain of virus used and the stock of rabbit employed were among the questions to be settled. The attempt reported in this thesis to adapt the hog cholera virus to rabbits failed, and, because of their great interest in the subject, the results were made available to other workers. Since the completion of this phase of the investigation, the efforts of other workers have been concentrated on the strain of virus found to be adaptable, and a lapinized vaccine is now being extensively studied. Ultraviolet irradiation failed to produce the desired results, but treatment of the virus with a nitrogen mustard did result in attenuation without loss of immunizing potency. A vaccine prepared by such a method should be studied in great detail in order to determine its merit, for the production of a non-virulent, antigenic virus is only the first step in the development of a desirable vaccine.

REVIEW OF LITERATURE

Most of the early studies of the nature of hog cholera were made by members of the United States Department of Agriculture. The impetus for the work came from the demands of the swine industry that such studies be carried out to determine its cause and appropriate methods of prevention. The cause of hog cholera was first incorrectly attributed by Salmon and Smith (53) to a microörganism now known as Salmonella choleraesuis (6). This organism was described by them as fatal to pigs, rabbits, guinea pigs, mice, and pigeons. Pigs would succumb to infection when the organisms were placed on their feed, or when they consumed the internal organs of swine dead from the disease. The role of Salmonella choleraesuis was questioned, however, when it failed to immunize pigs against hog cholera. Further doubt of the role of the Salmonella choleraesuis as a cause of hog cholera was raised when DeSchweinitz and Dorset (20) found that certain outbreaks of hog cholera occurred in which it was not the causative agent. These workers were able to transmit the disease by inoculation into healthy pigs of body fluids that had been freed of bacteria by filtration through porcelain filters. They concluded that all acute outbreaks were caused by some agent other than the hog cholera bacillus and that, in the chronic form of the disease, a mixed infectinn was present. McClintock, Boxmeyer, and Siffer (46) soon confirmed the work of DeSchweinitz and Dorset when they were able to produce the disease with bacteria-free filtrates prepared from tissues taken from hogs sick with cholera. They were also able to immunize hogs by the simultaneous injection of blood from sick hogs and blood from

hogs recovered from the disease. Dorset, Bolton, and McBryde (21) concluded from extensive studies that pure cultures of Salmonella choleraesuis produced only slight disturbances when injected subcutaneously, but that intravenous injections or feeding of the organisms often produced a severe illness. They found, moreover, that the illness produced by the organisms did not have the contagiousness of hog cholera and that hogs recovering from such illness were not immune to that disease. These workers also demonstrated that bacteria-free blood serum from affected hogs would regularly produce illness that possessed all the characteristics of the natural disease. Dorset, McBryde, and Niles (22) demonstrated that blood serum from immune hogs would protect non-immune hogs from an otherwise fatal dose of disease-producing blood. Confirmation of the early reports that the Salmonella choleraesuis did not pass the Pasteur-Chamberland filters F and B and certain Berkefeld filters was furnished by McBryde (44). He concluded from his studies that hog cholera was due to an ultra-visible virus sufficiently small to pass the pores of the filters used in the experiments.

The demonstration that hog cholera was caused by a virus and that immunization of pigs could successfully be accomplished by the simultaneous injections of immune serum and virus led to many studies of the efficacy of this immunizing procedure. The report of Dorset and Hess (23) is typical of many reports that appeared in the years immediately following the demonstration of the protective value of immune serum. These workers reported that of 234,136 hogs vaccinated, of which 36.5

per cent were sick at the time of treatment, a loss of only 13.1 per cent occurred. In 19,208 hogs vaccinated in uninfected herds only 49 pigs died. The use of the serum-virus method of immunizing hogs against hog cholera developed to enormous proportions. Simms' (55) report for the year 1949 shows the extent of use of this method. During 1949 the Bureau of Animal Industry supervised the production of approximately one and one-quarter billion cubic centimeters of the anti-hog cholera serum and approximately ninety-one million cubic centimeters of blood virus. This amount of virus, administered with the appropriate amounts of serum, was sufficient for immunizing forty-five million pigs weighing forty pounds each.

That the serum-virus method of immunizing against hog cholera obtained wide acceptance as the most effective means available of controlling the disease is indicated by the extent of its use. The method has its limitations, however, and Birch (7) summarized its contra-indications as follows: 1) in instances in which it cannot be applied by experienced men, 2) in a herd in which all animals cannot be immunized simultaneously, 3) in a herd in which treated animals cannot be properly segregated, 4) in suckling pigs, 5) in sows about to farrow, 6) in badly infected herds, and 7) in animals with a lowered resistance from any cause. The absence of immunizing agents with the effectiveness of the serum-virus method and without its limitations was, in a great measure, responsible for the extensive use of the serum-virus method. The lack of other immunizing agents was due primarily to inability to find a successful method of attenuating

the virus. McBryde and Cole (45) reviewed the failure of efforts to prepare hog cholera vaccines by the use of attenuating agents. Among the materials unsuccessfully tried were formalin, ammonia, chloroform, glycerin, and phenol. Graham (28) had very early found that heat could not be used to produce an attenuated virus capable of immunizing pigs. In spite of repeated failures to attenuate the virus, the need for an effective avirulent vaccine led to continued efforts to attenuate the virus, and in 1933 Boynton (8) reported the production of a successful vaccine. He inactivated the virus by treating it with eucalyptus oil. The lymph glands, spleen, and red bone marrow were employed in preparing the tissue vaccine, which came to be known as Boynton Tissue Vaccine, or simply B.T.V. Boynton (9) later reported on the use of the tissue vaccine in 2,600 pigs in which adequate protection was produced. By 1938 he was able to field-test the tissue vaccine extensively in thirty herds totalling 15,125 pigs (10). A negative record of hog cholera was obtained in these herds subsequent to use of tissue vaccine. B.T.V. was shown by Boynton, Woods, Wood, and Casselberry (11) to provide a satisfactory immunity for approximately twelve months when administered to healthy pigs, but pigs harboring bacterial infections at the time of vaccination either failed to develop a solid immunity or developed concurrent infection.

Shortly after the appearance of Boynton Tissue Vaccine, McBryde and Cole (45) described a vaccine prepared by treating virus-containing tissue with crystal-violet dye. Sixty-three lots of vaccine were prepared and tested on 271 pigs. Better than 98 per cent were adequately protected with the vaccine. The inadequacy of the early crystal-violet

vaccine was shown, however, by Munce (50), who noted that only 63.5 per cent of vaccinated pigs remained well when exposed to the virus. After further studies, Cole and McBryde (14) demonstrated that the crystal-violet vaccine would protect, for at least eight months, 89 per cent of pigs vaccinated at eight weeks of age and that the vaccine itself was incapable of spreading the disease. A still later report by Cole and Henley (16) showed that vaccinated pigs were protected against hog cholera for at least a year. They also demonstrated that if anti-hog cholera serum is administered simultaneously with the vaccine, or if the vaccine is administered within four weeks after serum treatment, it is ineffective. Potency of vaccine could not be related to strain of virus, virulence of virus, or the amount given to a pig whose tissues furnished material for preparation of the vaccine. Cole and Henley (17) confirmed their earlier work when they found that the crystal-violet vaccine did not interfere with the action of serum but that the serum consistently interfered with the immunogenic ability of the vaccine.

Both the crystal-violet and B.T.V. vaccines have been used rather extensively, in spite of their specific limitations. The extent to which these vaccines are used can be seen from a recent report by Simms (55). During the fiscal year 1949, approximately eleven million cubic centimeters of hog cholera vaccine were produced in establishments licensed by the Bureau of Animal Industry.

Eucalyptus oil and crystal-violet dye were the first chemical agents used that attenuated the virus without causing a complete loss of immunizing properties. The other chemical agents used either

failed to reduce the virulence of the virus or destroyed its immunizing powers. Recently, however, Tenbroek and Herriott (57) reported inactivation of several viruses, including the hog cholera virus, with nitrogen mustard. These workers inoculated 23 pigs with hog cholera virus in the form of both sera and organ suspensions that had been treated with a mustard. Challenge by pen exposure was done three weeks after the inoculations. Two of the vaccinated pigs died from causes other than hog cholera, and 3 died from cholera. The remaining 18 showed some immunity, but all developed some symptoms of hog cholera. This appears to be evidence that a third type of chemical agent is capable of attenuating the virus without total destruction of immunizing potency. These workers also obtained evidence that mustard-inactivated equine encephalomyelitis and rabies virus were immunogenic.

Among the early studies of the serum-virus method of immunizing pigs against hog cholera were unsuccessful attempts to pass the virus to animals other than swine. These studies were conducted in efforts either to attenuate the virus or to find some means of producing large amounts of immune serum. Inconclusive and non-repeatable results were obtained by King (40) and King and Wilson (41) when passage of the virus through sheep and horses was attempted. Craig (18) was unsuccessful in transmitting the virus through rabbits in succession, although he was able to recover virus from the carcasses of rabbits previously injected with fifteen cubic centimeters of a hog cholera blood filtrate. The insusceptibility to hog cholera of rabbits was also shown by Bohmer (original not seen) (49) and by Brockman (original not seen) (49), and of guinea pigs by Frohböse (original not seen)

(49). Passage of the virus through goats and peccaries by Roderick and Schalk (51) failed to adapt the virus to these animals or to attenuate the virus. The dog also proved non-susceptible to the virus, according to the work of Benner (5). This worker also failed to modify the virus through use of a modification of the Laidlow-Dunkin method of attenuating the dog distemper virus. Transmission experiments by Jacotot (original not seen) (49) in various mammals and birds, including guinea pigs, rabbits, and ferrets, showed that, although the virus could be recovered from the blood of these animals in from three to six days after injection, there was no clinical evidence of infection. More recently, Zichis (63) reported the passage of the hog cholera virus for ten transfers in sheep, but with no apparent diminuation in virulence. This author presented no evidence of propagation of the virus in the sheep. Muir (49) administered suspensions, prepared from spleens and brains of infected swine, by various routes, to mice, hamsters, ferrets, white rats, guinea pigs, and rabbits. Not one of the species used exhibited clinical symptoms or cerebral reactions of any practical value for diagnosis. In no species did the virus establish itself.

In addition to attempts to pass the virus to animals other than the pig, a search for natural vectors or animal reservoirs of the virus has been made. Dorset, McBryde, Niles, and Rietz (24) found the virus in none of the mammals and birds which they studied. The virus has been cultivated in vitro by Hecke (original not seen) (56), Tenbroeck (56), and Boynton, Takahashi, Woods, and Walker (12). No evidence

was presented by these workers that the virus was attenuated by propagation outside the body of the host pig.

All attempts to adapt the virus to a host other than the pig, to find natural reservoirs, or to attenuate the virus by animal passage failed, until Baker (4) succeeded in adapting it to rabbits. He was able to adapt the virus to the rabbit by using an alternate transfer technique that he had previously used in adaptation of the rinderpest virus to rabbits (3). One strain, after serial passage in rabbits, became attenuated for swine and fully immunized them to the virulent hog cholera virus. A second strain was not successfully adapted. No definite signs of illness were observed in the rabbits. Koprowski, James, and Cox (42) also attempted adaptation of the hog cholera virus to rabbits by use of the alternate transfer technique. They were able to pass the virus, a stock strain used for commercial production of serum and virus, through twelve generations of rabbits. A febrile response, lasting only 2-3 hours, was observed in some rabbits. Swine injected with the 8th and 9th rabbit passage died with typical hog cholera, and the 11th passage failed to be completely avirulent for swine.

The effect of ultraviolet light on many viruses has been the object of study by various workers. Some of these studies have been concerned with inactivation of the virus by ultraviolet irradiation for the purpose of producing a non-virulent vaccine. In some instances, attempts to attenuate viruses without destroying immunizing potency were successful.

McKinley and Holden (47) exposed the herpes virus to an Alpine sun lamp and succeeded in rendering it avirulent, but found that it also became non-antigenic for the rabbit. Galloway and Eidinow (26) destroyed the virulence of foot-and-mouth disease virus by exposing it for five minutes to the irradiations from a mercury vapor lamp. The yellow fever virus was completely inactivated by Gordon and Hughes (27), so that it was neither infectious nor antigenic. The virus was irradiated with a quartz mercury-arc lamp at a distance of 28 centimeters.

Hodes, Lavin, and Webster (34) presented evidence that the virulence of the rabies virus could be destroyed by ultraviolet irradiation for 45, 50, or 60 minutes without destruction of its immunizing power. They were able to obtain good protection in mice with the vaccine prepared by means of irradiation at 12.5 centimeters by a quartz mercury vapor lamp. Hodes, Webster, and Lavin (35) later concluded that the rabies virus exposed to the rays of a mercury vapor lamp under proper conditions loses virulence, yet retains considerable immunizing potency for mice. These workers studied the factors influencing the action of ultraviolet light and concluded that turbidity of solution, amount of shaking, kind of lamp, distance of material from lamp, and presence of organic material affected the results. They also found that rabies virus in a suspension not cleared by sedimentation and centrifugation was not destroyed by an exposure of 60 minutes.

After the first inactivation of the rabies virus by Hodes, Webster, and Lavin (35), Webster, and Casals (59, 60, 61, 62) developed the practicability of irradiated rabies vaccine in the prophylaxis of rabies in man and dogs. Habel (30) further studied the inactivation of

rabies virus by means of the Oppenheimer-Levinson lamp and exposure chamber. At the time of the report the details of the lamps were not released by the OSRD. Habel was able to prepare highly potent vaccines by the irradiation of rabies brain suspensions. The increased potencies of the vaccines depended, not on the shorter wave lengths of the new type of lamp, but upon the method of exposing the virus in the thin-film, continous-flow chamber that had been designed.

Two strains of influenza virus, PR8 and Melbourne, were inactivated by Salk, Lavin, and Francis (52) through the means of exposure to a quartz mercury vapor lamp for nine minutes. The inactivated virus was still capable of immunizing mice. The results of Henle and Henle (33) showed that ultraviolet irradiation did not alter the immunizing potency of certain strains of the influenza virus. The fluids containing the virus were exposed at a distance of seven inches, in open petri dishes, to irradiation by a General Electric Germicidal lamp. The dishes were shaken constantly during the exposure time.

A method of killing or inactivating bacteria and viruses in a fraction of a second was developed by Levinson, Milzer, Shaughnessy, Neal, and Oppenheimer and withheld from publication by the OSRD. In a paper published by these authors (43) they referred to the method and reported results of its use in inactivating various bacteria and viruses. The method consisted of exposure of the material in continuously flowing films less than one millimeter thick to the newly developed lamp, which they described as a powerful source of both total and extreme (below 2000 Angstroms) ultraviolet.

Havens, Watson, Green, Lavin, and Smadel (32) exposed suspensions of brains containing neurotropic viruses to a quartz mercury resonance lamp. The lamp operated at 30 milliamperes and 15,000 volts, with 85% radiation at 2537 Angstroms. The viruses of Saint Louis, Japanese, eastern and western encephalitis and of choriomeningitis were inactivated in 20 minutes of exposure.

Viruses, other than those infecting mammals, have been found to be inactivated by ultraviolet irradiation. Duggar and Hollandaer (25) inactivated the tobacco mosaic virus by wave lengths shorter than 311 Angstroms. The Rous chicken sarcoma virus was found by Baker and Peacock (2) to be destroyed by ultraviolet irradiation. They calculated that the active agent in the form of tumor tissue suspension would survive an exposure of at least five times that necessary to kill most pathogenic bacteria. They postulated that the small size of the active particles may be the chief factor in determining this difference in susceptibility. Another virus affecting chickens, the fowl pox virus, was inactivated by Graham, Brandly, and Levine (29) by exposure of the virus for two hours to irradiations from a mercury vapor lamp. Exposures for 15 to 90 minutes attenuated the virus.

The effectiveness of various lamps for irradiation was studied by Buttolph (13). He stated that the low-pressure mercury arcs in recently developed glass tubes, transmitting about 65 per cent of the ultraviolet light at wave lengths of 2537 Angstrom units, provide an over-all efficiency about five times that of quartz lamps. Buttolph further suggested that an ultraviolet intensity of about 0.01 watt

per square foot of surface produced complete killing of bacteria in eight to ten minutes.

Hollaender, Oliphant, and Andrews (36) in a study of the effect of irradiations on virus found that most viruses inactivated by ultraviolet light show a maximum sensitivity at 2650 Angstrom units, which is close to the nucleic acid absorption band. They state that for adequate irradiation the material must be free of dense aggregates and that all particles must receive adequate irradiation. Earlier, Arnow (1), in reviewing the nature of irradiation, stated that the protein change is a denaturation occurring in two steps. In the first step, or preliminary process, the protein molecule becomes a new chemical entity, after which, the physical process, the formation of a visible coagulum, takes place.

The literature revealed no evidence that the hog cholera virus had been subjected to ultraviolet irradiation, although some of the work on the irradiation of viruses suggests that it might be an effective means of attenuation.

EXPERIMENTAL

Objectives

The objects of the investigation were to modify the hog cholera virus by adaptation to the rabbit, irradiation with ultraviolet light, or exposure to nitrogen mustard, and to determine whether the modified virus was avirulent and at the same time antigenic.

Materials

Strain of Virus

The strain of virus employed was obtained from a commercial firm that used it for the routine production of hog cholera virus, vaccine, and immune serum. The virus was used by the firm under the supervision of the Bureau of Animal Industry, United States Department of Agriculture, and it met the specifications set by that agency for strains of virus used in the manufacture of the biologicals named. At no time during the course of these studies was there reason to believe that the virus was not the standard stock virus or that it contained any variant strains.

Experimental Animals

Pigs of weaning age (8-10 weeks) were obtained either from the V.P.I. Animal Husbandry Department or from a swine producer in Shenandoah County, Virginia. The farms from which the pigs came had no history of recent cholera outbreaks, and none occurred during the period in which the pigs were being purchased. The swine herds were also free from other bacterial and viral diseases.

The pigs obtained from the V.P.I. Animal Husbandry Department were farrowed by sows that had previously been immunized against hog cholera by the serum-virus method, while the pigs purchased from Shenandoah County came from a non-vaccinated herd.

The pigs, prior to inoculations, were kept in an isolated stock pen equipped with a small shelter and the necessary watering and feeding devices. They were moved from the stock pen to stalls or cages in an animal barn prior to inoculation. The stalls had smooth concrete floors that drained forward to a gutter outside the stall. The gutter was of a width and depth that prevented the passage of excreta from stall to stall or gutter to stall. The back wall of the stalls was of cinderblock, while the front was of an open, stanchion-type construction. The stalls were separated from each other at the floor level by a concrete strip about six inches in width and height. The side walls, of hardwood construction, approximately six inches wide and 60 inches high, were built on top of the concrete strips.

The cages had slatted hardwood floors, sides and backs of sheet metal, and a door and top of woven wire construction. The cages were on legs that raised the bottom approximately one foot above the concrete floor. All excreta drained to a gutter in front of the cages. Each stall and cage was equipped with the necessary feed and water containers.

The rabbits used in the rabbit passage of the virus came from the colony maintained at the Virginia Agricultural Experiment Station. They were of a mongrel stock kept for experimental

and diagnostic purposes. Young, vigorous rabbits of five to six pounds in weight were selected for use. At the time of inoculation they were moved to separate cages.

Nitrogen Mustard

The nitrogen mustard used was methyl-bis (β-chloroethyl)-amine. It was obtained through the courtesy of Dr. E. P. Johnson, Virginia Agricultural Experiment Station.

Ultraviolet Lamps

Two lamps, manufactured by the Westinghouse Electric Corporation, were used. One was designated by the manufacturer as CH4. This lamp is a 100 watt mercury vapor lamp with an outer bulb of #772 glass. It transmits no radiation below 3050 Angstrom units, and is equipped with a black light filter, which limits the radiation transmitted to ultraviolet light of predominantly 3650 Angstrom units.

The second lamp was designated by the manufacturer as WL793. It is equipped with a built-in transformer and is described as a Cold Cathode Germicidal Sterilamp. Radiations by this lamp are generated by an electrical discharge through low pressure inert gases and mercury vapor enclosed within the special glass tube of the lamp. Approximately 95 per cent of the ultraviolet is in the region of 2537 Angstrom units. The WL793 lamp operates on approximately 105 volts A.C., with an operating current range in amperes of 0.040 - 0.045. The ultraviolet intensity (2537AU) in microwatts per square centimeter at one meter is 3, and the ultraviolet output (2537AU) in watts is 0.27.

Methods

Care of Experimental Animals

At the time of delivery, the pigs were placed in the isolated stock pen. All pigs were kept in the stock pen for at least two weeks prior to their use in the experiments. This quarantine was imposed to insure their freedom from hog cholera and other infectious diseases, and to allow time for any passive immunity acquired from their dams to disappear. During the first week after receipt, the pigs were given sodium fluoride for removal of ascarids.

The ration consisted of a commercial hog feed which contained 15% protein and the additional nutrients necessary for proper growth.

Before inoculation, the pigs were moved from the stock pen to stalls or cages in the animal barn. Rigid control measures were used to prevent the spread of infection in the barn and from the barn to the stock pigs. The feed for the latter was stored in a separate building from that which held the feed for the inoculated pigs. The feeding, watering, and handling of the pigs in the stock pen were done by a person who had no contact with pigs removed from the stock pen for inoculation purposes.

Prevention of spread of infection in the barn was accomplished by preventive measures fitted to the needs of each group of pigs placed in the barn. The feeding and watering were done from outside the stall or cage from containers that were not allowed to come in contact with any object in contact with the pigs. The

stalls were entered by the caretaker only when it was necessary to remove litter, after which his boots and hands were thoroughly washed with a disinfectant before a second stall was entered. The equipment, such as shovels, was thoroughly washed and disinfected after use in each stall or cage. The caretaker avoided any bodily contact with the pigs so that the virus, when present, was not picked up on the hands or clothing.

The person taking the temperatures of the pigs wore coveralls and rubber boots. His hands and boots were thoroughly washed and disinfected before and after entering each stall. If the coveralls were suspected of becoming contaminated, they were changed for clean ones before another stall or cage was entered.

As soon as the pigs were removed from the barn at the end of an experiment, the floor and walls of the stalls and cages were washed thoroughly with soap and water several times before being thoroughly scrubbed with an alkali solution. The feed and water troughs were autoclaved after being washed. All litter and refuse were burned.

Control pigs used to detect the presence of the virus in the various suspensions were always placed in stalls or cages separate from those of the pigs injected with suspensions that had received attenuating treatment.

Criteria for Results

Daily observations were made of the inoculated animals for the development of the following symptoms described by Birch (7) and Udall (58) as indicative of the disease: fever (see Figure 1),

depression, anorexia, motor irritation or stiffness and incoordination, conjunctivitis, constipation followed by diarrhea, and discoloration of the skin due to congestion or hemorrhage.

Complete autopsies were performed on all animals when they were necessary to establish the presence of the disease. The following pathological changes, described by Birch (7) and Udall (58), were searched for, as well as any additional changes that might be present: petechial hemorrhages beneath the kidney capsule; petechial hemorrhages in the mucosa of the bladder, larynx, and trachea; larger hemorrhages in the intestinal mucosa, lungs, epicardium, and spleen; and peripheral hemorrhages of the lymph glands (see Figures 2, 3, and 4).

A diagnosis of hog cholera was made when the appearance of typical symptoms was associated with the pathological changes generally accepted as the results of infection by hog cholera virus. In those instances where any doubt of the presence of the disease existed, filtrates were prepared from the spleen or blood and injected into susceptible pigs. Such check pigs were then observed for the development of symptoms and lesions. In this manner any confusing bacterial or viral infection was differentiated from hog cholera.

Rabbit Passage of the Virus

Two milliliters of the stock virus were injected intramuscularly into a susceptible pig that had been moved from the stock pen to the barn. This pig was designated as a virus pig. Daily obser-

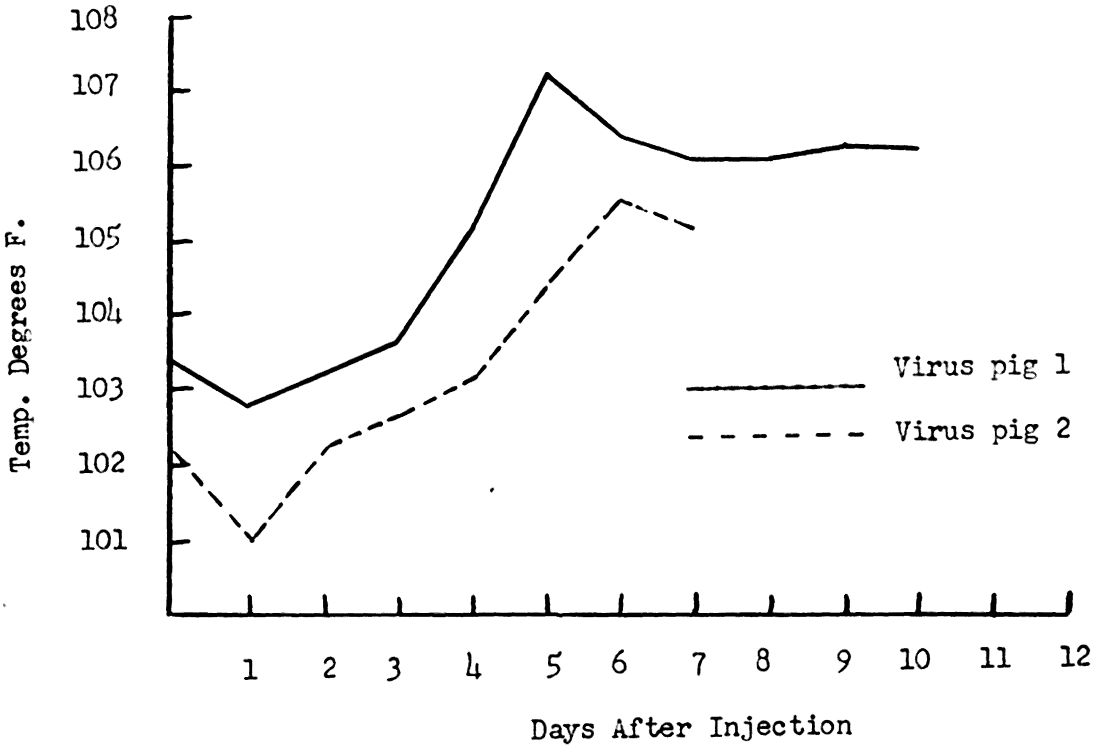

Figure 1. Temperature curves of pigs injected intramuscularly with hog cholera virus.

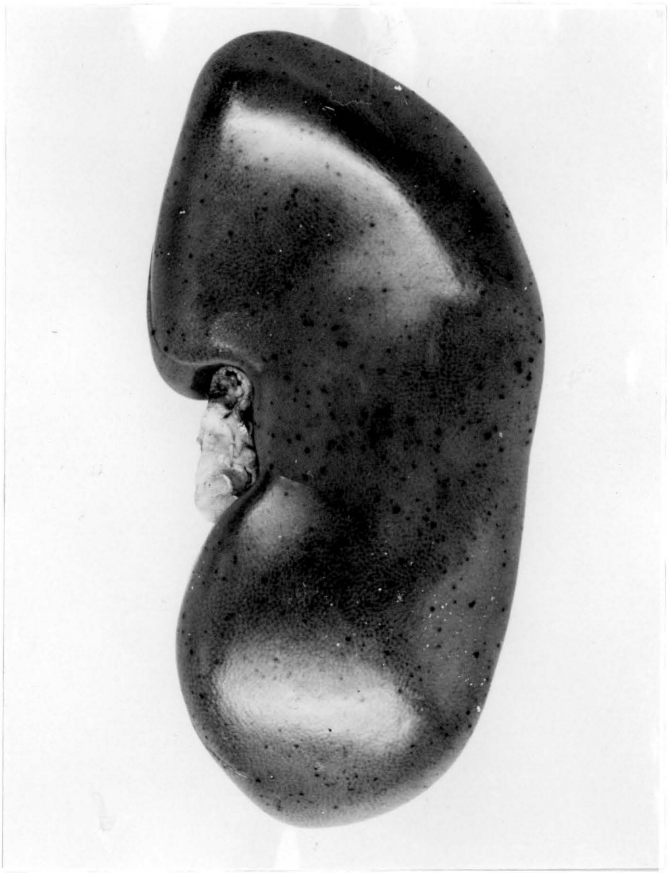

Figure 2. Petechial hemorrhages in the kidney of a pig dying from hog cholera.

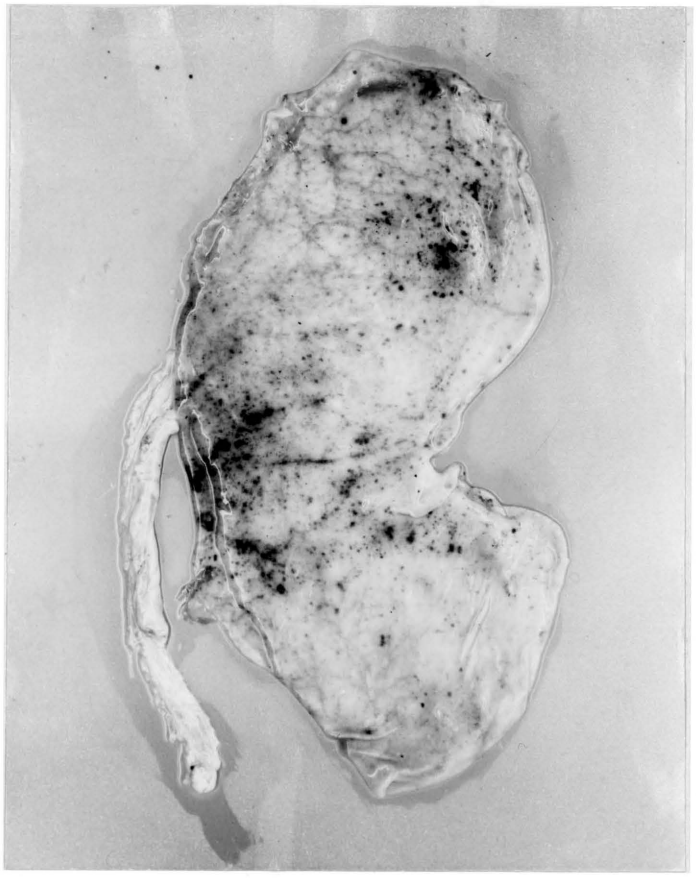

Figure 3. Hemorrhages in the gastric mucosa of a pig dying from hog cholera.

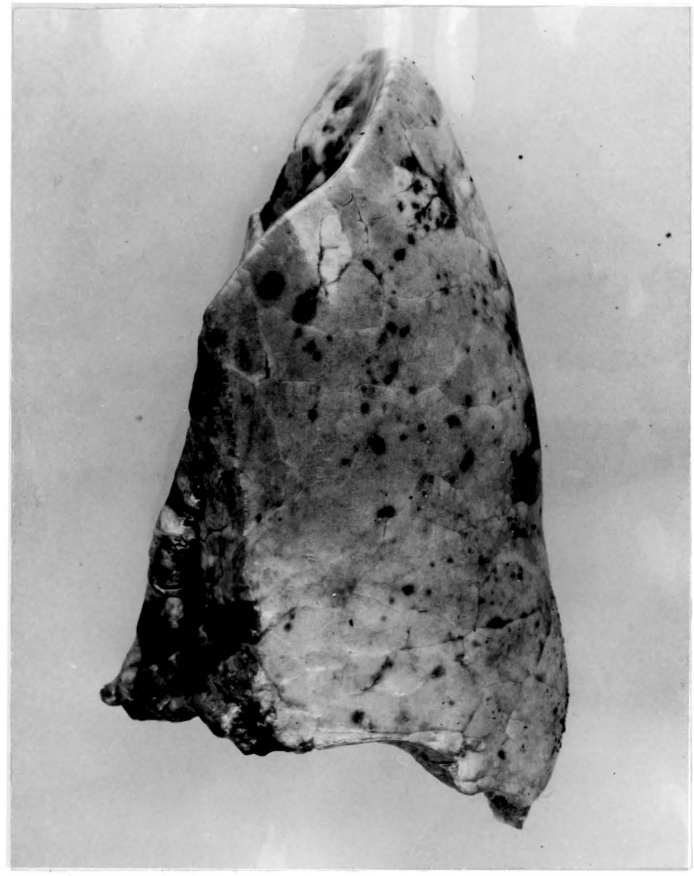

Figure 4. Hemorrhages in the lungs of a pig dying from hog cholera.

vations of symptoms were made, and, when the temperature rise reached the peak, the pig was sacrificed. The spleen was removed under aseptic conditions, and a portion was ground in a sterile mortar with sufficient sterile physiological saline to make a 10 per cent suspension of spleen tissue. The large particles were removed by filtration through sterile gauze, and the suspension further clarified by centrifugation at 1800 rpm for five minutes. The supernatant fluid prepared in this manner was designated as pig-spleen virus.

Two milliliters of the pig-spleen virus were injected into the marginal ear vein of a rabbit that had been removed from the stock pen to an isolation cage. Observations were made at two- to four-hour intervals, and the rabbit sacrificed between the 72nd and 96th hour after inoculation. Careful examination was made of the rabbit for the presence of lesions, after the spleen had been removed aseptically. A suspension was prepared from the rabbit spleen in the manner previously described for the preparation of the pig-spleen virus. This suspension was designated as the first alternate passage rabbit-spleen virus.

Two milliliters of the first alternate passage rabbit-spleen virus were injected intramuscularly into a pig, from which a pig-spleen virus was prepared for injection into a rabbit. This rabbit, after 72 to 96 hours, furnished the second alternate passage rabbit-spleen virus for inoculation. This alternation of passages from the pig to the rabbit was continued for six

passages. After the sixth alternate passage, the virus was carried serially in rabbits. This was accomplished by preparing rabbit-spleen virus in the manner already described. After the intravenous injection of the rabbit-spleen virus, frequent observations were made for the development of symptoms by the rabbits. Careful examinations for pathological changes were made at the time of autopsy, 72 to 96 hours after the inoculation.

At each serial passage, a two-milliliter portion of the rabbit-spleen virus was injected intramuscularly into a susceptible pig. These pigs served to detect the presence of the hog cholera virus in the rabbit-spleen preparations. The appearance of typical symptoms and lesions proved the presence of virulent virus in the rabbit-spleen suspensions. The pig that failed to develop symptoms of cholera was challenged after three to four weeks with two milliliters of stock virus, which was also checked for virulence at the same time. The challenge determined whether the pig injected with the rabbit-spleen virus had been immunized by it. The presence of immunity in the pig would have furnished evidence of modification associated with retention of immunizing properties. The scheme for the alternate and serial passage of the virus is shown in figure 5.

A single attempt was made to determine the infectiousness of the blood of a rabbit that had been injected with pig-spleen virus. Two milliliters of blood were withdrawn from Rabbit 5a on the third day and again on the fourteenth day after injection with

the fifth passage rabbit-spleen virus. On each of these days a susceptible pig was injected with the sample of blood taken and observed for development of symptoms and lesions.

Ultraviolet Irradiation of the Virus

Limitation of barn space for the isolation of the test and control pigs made it necessary to conduct this part of the studies in a series of identical experiments. The pig-spleen virus for irradiation was prepared from a new virus pig that had been infected with either the stock virus or pig-spleen virus on hand from the previous experiment. The first experiment was conducted with the Westinghouse Lamp CH4, while the WL793 lamp was used for the remainder of the experiments.

Two milliliters of stock virus were injected intramuscularly into a susceptible pig. This pig, designated as a virus pig, was sacrificed when the temperature rise reached the peak. The spleen was removed aseptically, and a portion of it was ground in a sterile mortar. Sufficient sterile physiological saline was added to make a final suspension of 10 grams of splenic tissue in 100 milliliters of saline. Large particles were removed by filtering the suspension through sterile gauze, and the filtrate was further clarified by centrifugation at 1800 rpm for five minutes. The supernatant fluid, designated as pig-spleen virus, was decanted into sterile bottles and either used immediately or stored at 4° C. until used.

The pig-spleen virus was poured into a sterile petri dish to a depth of one to two millimeters. The dish was then placed 15 centimeters from the ultraviolet lamp. The dish was placed

on a tray of ice to prevent excessive evaporation during the period of irradiation. It was constantly shaken during the period of exposure so that the pig-spleen virus was continuously being mixed. At the completion of the irradiation, the pig-spleen virus, now designated as irradiated pig-spleen virus, was poured into sterile bottles.

Test of the irradiated pig-spleen virus was accomplished by the intramuscular injection of five milliliters into susceptible pigs isolated in the barn. Control pigs were injected at the same time with a five milliliter portion of the non-irradiated pig-spleen virus. Daily observations were made for the development of symptoms in all pigs, and autopsies were performed in all instances requiring post-mortem examination. All pigs that survived the injection of the irradiated pig-spleen virus were challenged at the end of three to four weeks. The challenge was made by the intramuscular injection of two milliliters of the stock virus. At the same time, susceptible pigs were injected with two milliliters of the stock virus used for the challenge, as a check on its infectiousness.

Nitrogen Mustard Treatment of the Virus

Limitation of barn space for the proper isolation of the pigs made it necessary to conduct this part of the studies in a series of experiments. In the first series the blood from a virus pig, designated as blood virus, was used for treatment by the nitrogen mustard. The blood was obtained, aseptically, from the virus

pig at the height of the temperature rise. The infection had been induced in the virus pig by the injection of the stock virus or pig-spleen virus on hand from previous work. Coagulation of the blood was prevented by sodium citrate.

Five milliliters of the blood virus were added to a dry, sterile vial containing ten milligrams of the mustard. The vial was shaken for 30 minutes and then allowed to stand for an additional 30 minutes. At the end of the hour's treatment five milliliters of the blood virus, now designated as mustard blood virus, were injected intramuscularly into susceptible pigs. Control pigs were used to determine the infectiousness of the untreated blood virus.

Pig-spleen virus was used in the remainder of the experiments. Two milliliters of stock virus were injected into a susceptible pig. This pig, designated as a virus pig, was sacrificed when the temperature rise reached the peak. The spleen was removed aseptically and a portion of it ground in a sterile mortar. Sufficient sterile physiological saline was added to make a final suspension of 10 grams of splenic tissue in 100 milliliters of saline. Large particles of tissue were removed by filtering the suspension through sterile gauze. The filtrate was further clarified by centrifugation at 1800 rpm for five minutes. The supernatant fluid, designated as pig-spleen virus, was decanted into sterile bottles and either used immediately or stored at 4° C. until needed.

Treatment of the pig-spleen virus was accomplished by adding five milliliters of the pig-spleen virus to a dry, sterile, stoppered vial containing ten milligrams of the nitrogen mustard. The

vial was mechanically shaken for 30 minutes and then allowed to stand for an additional 30 minutes. Five milliliters of the treated virus, now designated as mustard pig-spleen virus, were injected intramuscularly into susceptible pigs at the end of the hour's treatment, except in Experiment M6, when the injections were made 24 hours after the material was prepared.

Control pigs, used to determine the infectiousness of the untreated pig-spleen virus, were used in all experiments. Daily observations were made for the development of symptoms, and autopsies were performed in those instances requiring post-mortem examinations for the interpretation of results.

All pigs that survived the injection of the mustard pig-spleen virus were challenged after three to four weeks after inoculation. The challenge was made by the intramuscular injection of two milliliters of the stock virus, except in Experiment M7 in which pen exposure to sick pigs was used. At the time of challenge, susceptible pigs were injected with two milliliters of the stock virus as a check on its infectiousness.

Results

Rabbit Passage of the Virus

The hog cholera virus was passed from pig to rabbit for six alternate passages, after which the virus was passed successfully for five serial passages through rabbits. The virus could not be demonstrated in the rabbit-spleen suspension prepared for the sixth serial rabbit passage. The results are shown schematically in Figure 5.

There was no evidence of attenuation in virulence of the virus for the pig during the alternate passages and the first five serial passages of the virus in rabbits. Typical temperature curves of pigs injected with alternate passage rabbit-spleen virus and serial passage rabbit-spleen virus are shown in Figure 6.

No evidence of adaptation of the virus to rabbits was obtained. The rabbits used in the alternate and serial passages did not show any symptoms attributable to the virus, nor did they show any lesions on post-mortem examination. Typical temperature curves obtained in injected rabbits are shown in Figure 7.

A pig injected with two milliliters of blood drawn from rabbit 5a three days after the rabbit had been injected with pig-spleen virus from pig 5 developed typical symptoms and lesions. The pig injected with two milliliters of blood drawn from rabbit 5a 14 days after injection of the rabbit did not develop symptoms. This pig developed typical symptoms and lesions when challenged with virulent virus. The results are shown schematically in Figure 8.

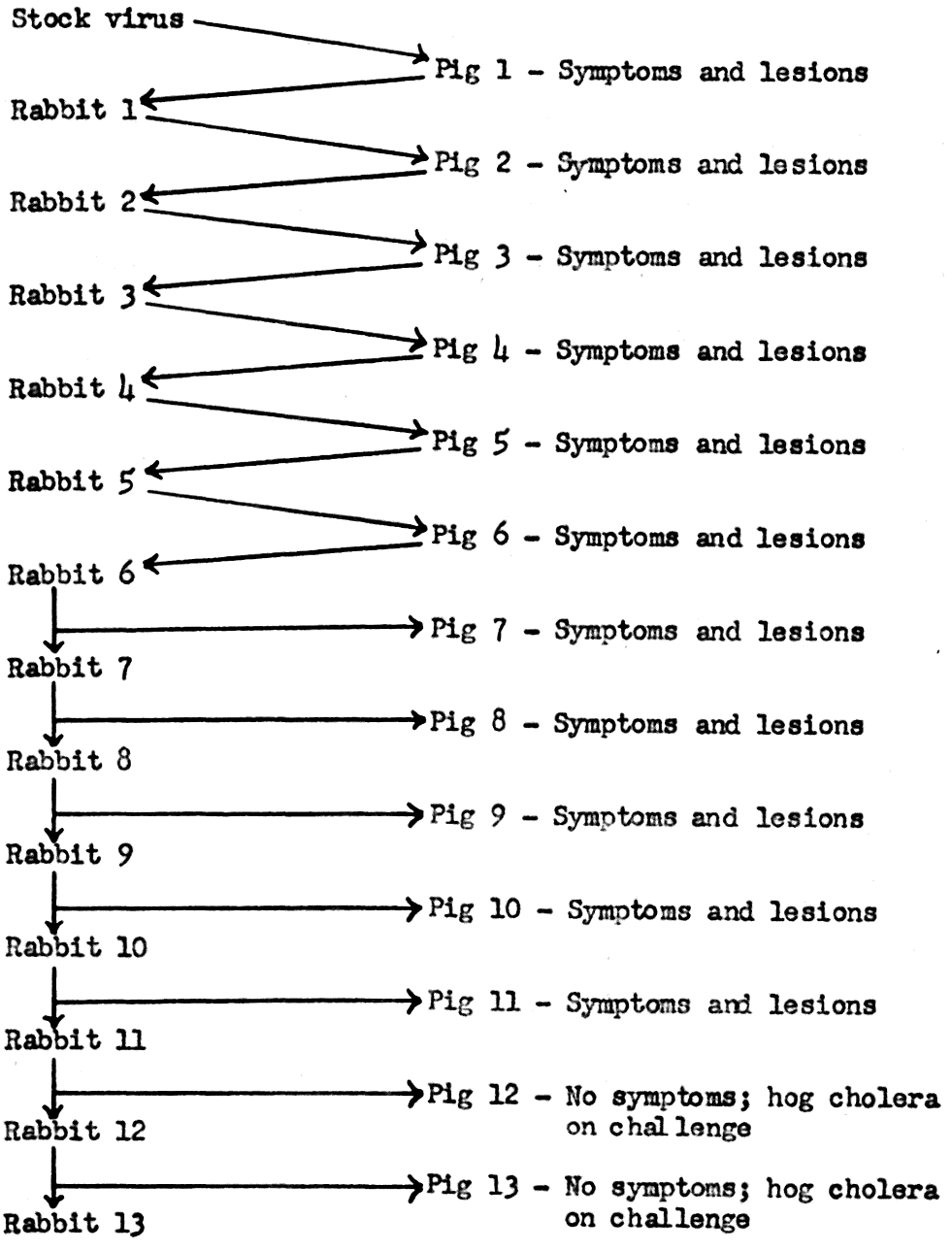

Figure 5. Summary of the alternate and serial passages of the hog cholera virus in rabbits.

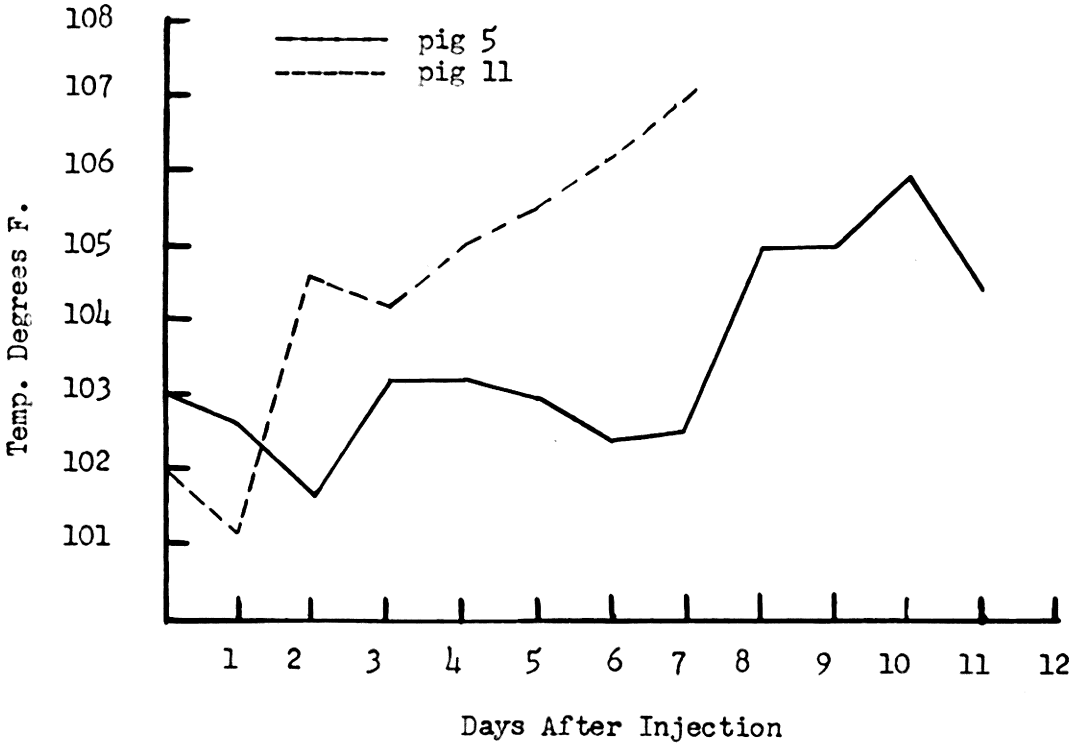

Figure 6. Temperature curves of pigs 5 and 11, injected with rabbit-spleen virus from rabbits 4 and 10 respectively.

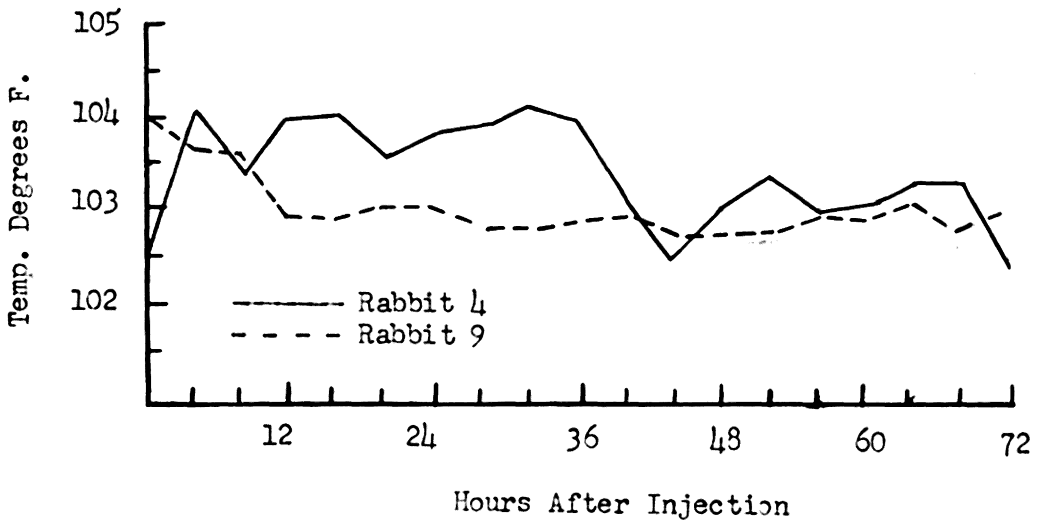

Figure 7. Temperature curves of rabbits injected with the 4th alternate and the 3rd serial rabbit passage rabbit-spleen virus .

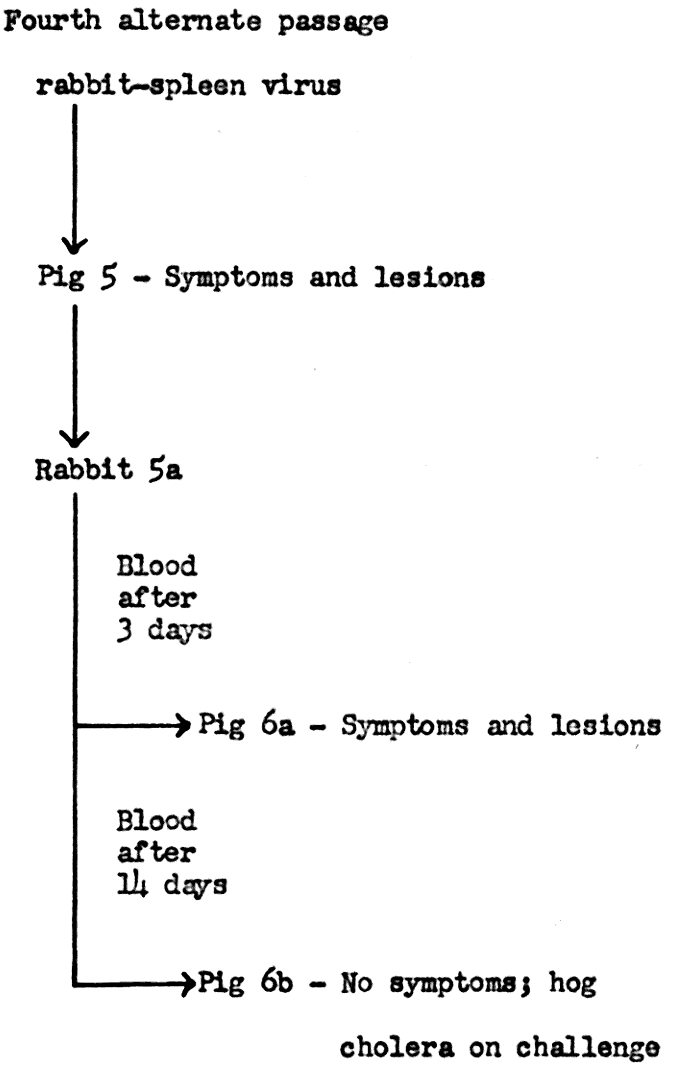

Figure 8. Survival of the hog cholera virus in the rabbit.

Ultraviolet Irradiation of the Virus

Ultraviolet irradiation of the hog cholera virus with a Wastinghouse lamp CH4, for 30 and 60 minutes, failed to attenuate the virus consistently for pigs. The results are shown in Table 1. Typical temperature curves obtained in pigs injected with pig-spleen virus irradiated by lamp CH4 are shown in Figure 9.

Ultraviolet irradiation of the hog cholera virus with a Westinghouse lamp WL793, for 15 and 30 minutes, failed to attenuate the virus for pigs. Only three pigs, of a total of 17 pigs injected with pig-spleen virus irradiated for 60 minutes by the WL793 lamp, failed to develop hog cholera. These three pigs were immune when challenged with virulent virus. The results of irradiation of pig-spleen virus by lamp WL793 are shown in Tables 2 through 5 and summarized in Table 6. Typical temperature curves of pigs injected with pig-spleen virus irradiated with lamp WL793 are shown in Figure 10.

TABLE 1

Effects on Pigs of Spleen Virus Irradiated by Lamp CH4

Experiment UV1

Pig no.	Irradiation, time, minutes	Results of injection		Results of challenge
		Symptoms	Lesions	
UV1	30	Yes	Yes	-
UV2	60	No	-	Immune
UV3	30	Yes	Yes	-
UV4	30	Yes	Yes	-
UV5	60	Yes	Yes	-
UV6	60	Yes	Yes	-
UVC1[a]	0	Yes	Yes	-
UVC2[a]	0	Yes	Yes	-
UVC3[b]	0	Yes	Yes	-

(a) Controls for non-irradiated virus.

(b) Control for challenge virus.

TABLE 2

Effects on Pigs of Spleen Virus Irradiated by Lamp WL793

Experiment UV2

Pig no.	Irradiation, time in minutes	Results of injection		Results of challenge
		Symptoms	Lesions	
UV7	30	Yes	Yes	-
UV8	30	Yes	Yes	-
UV9	30	Yes	Yes	-
UV10	15	Yes	Yes	-
UV11	15	Yes	Yes	-
UV12	15	Yes	Yes	-
UVC4(a)	0	Yes	Yes	-

(a) Control for non-irradiated virus.

TABLE 3

Effects on Pigs of Spleen Virus Irradiated by Lamp WL793

Experiment UV3

Pig no.	Irradiation, time in minutes	Results of injection		Results of challenge
		Symptoms	Lesions	
UV13	60	Yes	Yes	-
UV14	60	Yes	Yes	-
UV15	60	Yes	Yes	-
UV16	60	Yes	Yes	-
UV17	60	Yes	Yes	-
UVC5[a]	0	Yes	Yes	-

(a) Control for non-irradiated virus.

TABLE 4

Effects on Pigs of Spleen Virus Irradiated by Lamp WL793

Experiment UV4

Pig no.	Irradiation, time in minutes	Results of injection		Results of challenge
		Symptoms	Lesions	
UV18	60	Yes	-	Immune
UV19	60	No	-	Immune
UV20	60	Yes	-	Immune
UV21	60	Yes	Yes	-
UVC6[a]	0	Yes	Yes	-
UVC7[b]	0	Yes	Yes	-

(a) Control for non-irradiated virus.

(b) Control for challenge virus.

TABLE 5

Effects on Pigs of Spleen Virus Irradiated by Lamp WL793

Experiment UV5

Pig no.	Irradiation, time in minutes	Results of injection		Results of challenge
		Symptoms	Lesions	
UV22	60	Yes	Yes	-
UV23	60	Yes	Yes	-
UV24	60	Yes	Yes	-
UV25	60	Yes	Yes	-
UV26	60	Yes	Yes	-
UV27	60	Yes	Yes	-
UV28	60	Yes	Yes	-
UV29	60	Yes	Yes	-
UVC8[a]	0	Yes	Yes	-

(a) Control for non-irradiated virus.

TABLE 6

Summary of Effects on Pigs of Irradiated Virus

Experiments UV1 through UV5

No. of pigs	Irradiation		Effect of virus		Immune to challenge
	Lamp	Min.	Symptoms	Lesions	
3	CH4	30	3	3	0
3	CH4	60	2	2	1
3	WL793	15	3	3	0
3	WL793	30	3	3	0
17	WL793	60	16	14	3
8(a)	-	0	8	8	-

(a) Controls for non-irradiated virus.

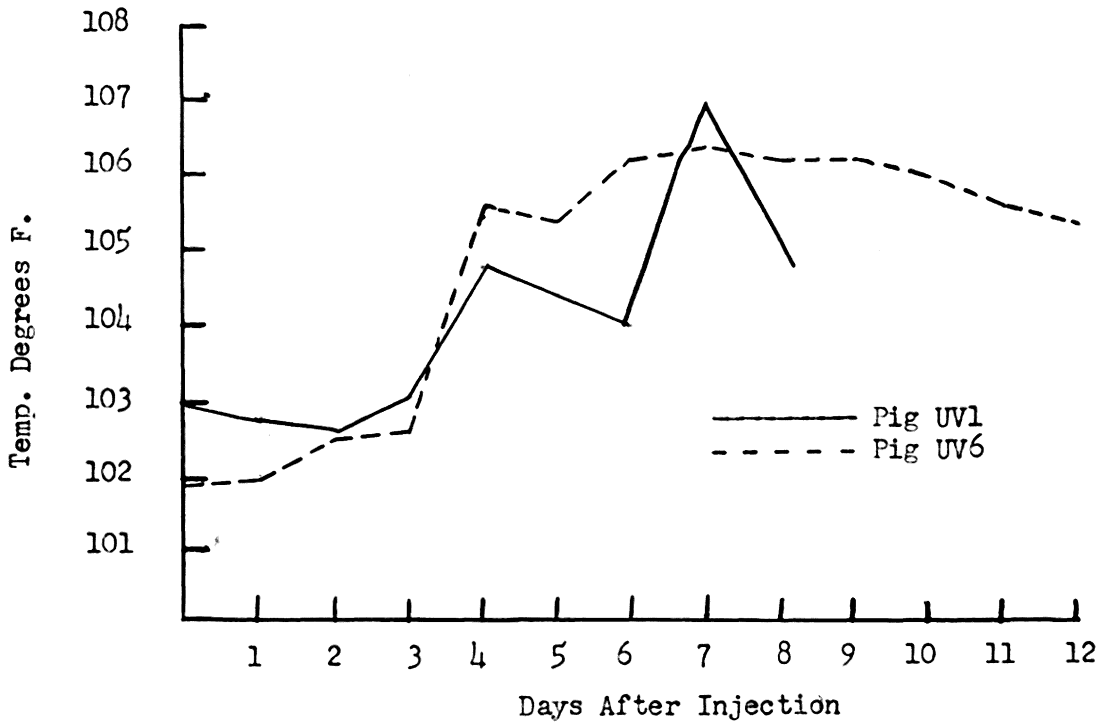

Figure 9. Temperature curves of pigs UV1 and UV6, injected with pig-spleen virus irradiated with lamp CH_4 for 30 and 60 minutes respectively.

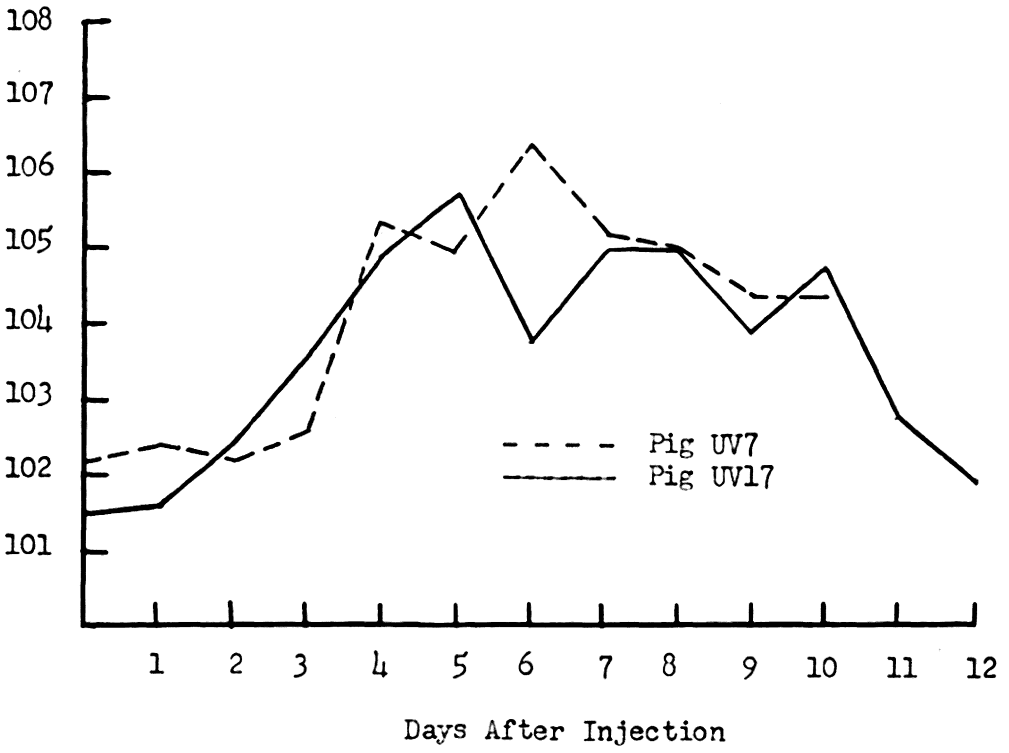

Figure 10. Temperature curves of pigs UV7 and UV17, injected with pig-spleen virus irradiated with lamp WL793 for 30 and 60 minutes respectively.

Nitrogen Mustard Treatment of the Virus

Nitrogen mustard either failed to reduce the pathogenicity of the hog cholera virus used in the form of blood virus or destroyed its virulence as well as its immunizing potency. Four pigs injected with mustard blood virus developed typical symptoms and lesions of hog cholera. Two pigs failed to develop symptoms of the disease when injected with mustard blood virus, but were found not to be immune when challenged with virulent virus. The results of the injection of pigs with mustard blood virus are shown in Table 7.

Nitrogen mustard attenuated the hog cholera virus, in the form of pig-spleen virus, for the pig. The results of injection of mustard pig-spleen virus into pigs are shown in Tables 8 through 13 and summarized in Table 14. No visible symptoms of hog cholera appeared in 32 pigs injected with mustard pig-spleen virus, although seven pigs developed a slight transitory rise in temperature after the injection. The rise in temperature occurred in one pig on the fifth day, and in the other six on or after the twelfth day following injection (Experiment M4, Table 10). The rise in temperature in the six pigs on or after the twelfth day could not be associated with the presence of the virus. Four of the six pigs in which a temperature rise was observed on or after the twelfth day died before challenge. These pigs had extensive damage to the liver, including abscesses, from a previous ascarid infection, and were also parasitized with lung worms, which produced a verminous pneumonia.

The other two pigs developing a fever on the twelfth day or later had concurrent symptoms of pneumonia, from which they rapidly recovered.

Eight of the 32 pigs injected with mustard pig-spleen virus died before challenge with virulent virus. In addition to the four pigs of Experiment M4 already noted, two others of this experiment died from the same causes. One pig in Experiment M2 (Table 8) died from an intestinal obstruction, and one pig in Experiment M3 (Table 9) died from cystitis, making a total of eight pigs lost before challenge. None of the eight pigs dying before challenge showed any symptoms or lesions of hog cholera, nor could the virus be recovered from tissues taken at autopsy.

Nitrogen mustard did not destroy the immunizing properties of the hog cholera virus when it was used in the form of pig-spleen virus. The results of the challenge of the pigs injected with mustard pig-spleen virus are shown in Tables 8 through 13, and summarized in Table 14. No symptoms of cholera were observed in 19 pigs injected with two milliliters of virulent virus three to four weeks after they had received five milliliters of mustard pig-spleen virus. Only four of the 19 pigs showed a transitory temperature rise following the challenge. This rise in temperature occurred on the third or fourth day, and in no instance did it go above 105° F. Typical temperature curves of the pigs after injection with mustard pig-spleen virus and after challenge are shown in Figure 11.

Five pigs injected with five milliliters each of mustard pig-spleen virus were immune when challenged by pen exposure (Table

13). No symptoms developed in these five pigs.

Non-injected pigs in contact with pigs injected with mustard pig-spleen virus did not contract the disease. Three pigs in pen contact with five pigs injected with five milliliters each of mustard pig-spleen virus remained normal. Two of these pigs developed typical hog cholera when injected with virulent virus, and the third contracted the disease through contact.

TABLE 7

Effects on Pigs of Mustard-Treated Blood Virus.

Experiment M1

Pig no.	Virus lot	Results of injection			Results of challenge
		Symptoms		Lesions	
		Temp.	Visible		
M1	1	No	No	No	Hog cholera
M2	1	No	No	No	Hog cholera
M3	2	Yes	Yes	Yes	-
M4	2	Yes	Yes	Yes	-
M5	2	Yes	Yes	Yes	-
M6	2	Yes	Yes	Yes	-
MC1[a]	1	Yes	Yes	Yes	-
MC2[b]	2	Yes	Yes	Yes	-
MC3[c]	-	Yes	Yes	Yes	-

(a) Control for untreated virus, lot 1.

(b) Control for untreated virus, lot 2.

(c) Control for challenge virus.

TABLE 8

Effects on Pigs of Mustard-Treated Spleen Virus

Experiment M2

Pig no.	Results of injection			Results of challenge
	Symptoms		Lesions	
	Temp.	Visible		
M7	No	No	No	Immune
M8	No	No	No	(a)
M9	No	No	No	Immune
M10	No	No	No	Immune
MC5(b)	Yes	Yes	Yes	-
MC6(c)	Yes	Yes	Yes	-

(a) Died from causes other than hog cholera.

(b) Control for untreated virus.

(c) Control for challenge virus.

TABLE 9

Effects on Pigs of Mustard-Treated Spleen Virus

Experiment M3

Pig no.	Results of injection			Results of challenge
	Symptoms		Lesions	
	Temp.	Visible		
M11	No	No	No	Immune
M12	No	No	No	(a)
M13	No	No	No	Immune
M14	Yes	No	No	Immune
M15	No	No	No	Immune
MC8(b)	Yes	Yes	Yes	-
MC9(c)	Yes	Yes	Yes	-

(a) Died from causes other than hog cholera.

(b) Control for untreated virus.

(c) Control for challenge virus.

TABLE 10

Effects on Pigs of Mustard-Treated Spleen Virus

Experiment M4

Pig no.	Results of injection			Results of challenge
	Symptoms		Lesions	
	Temp.	Visible		
M16	Yes	No	No	(a)
M17	No	No	No	(a)
M18	Yes	No	No	(a)
M19	No	No	No	(a)
M20	Yes	No	No	(a)
M21	Yes	No	No	(a)
M22	Yes	No	No	Immune
M23	Yes	No	No	Immune
MC10[b]	Yes	Yes	Yes	-
MC11[c]	Yes	Yes	Yes	-

(a) Died from causes other than hog cholera.

(b) Control for untreated virus.

(c) Control for challenge virus.

TABLE 11

Effects on Pigs of Mustard-Treated Spleen Virus

Experiment M5

Pig no.	Results of injection			Results of challenge
	Symptoms		Lesions	
	Temp.	Visible		
M24	No	No	No	Immune
M25	No	No	No	Immune
M26	No	No	No	Immune
M27	No	No	No	Immune
M28	No	No	No	Immune
M29	No	No	No	Immune
M30	No	No	No	Immune
MC12(a)	Yes	Yes	Yes	-
MC13(b)	Yes	Yes	Yes	-

(a) Control for untreated virus.

(b) Control for challenge virus.

TABLE 12

Effects on Pigs of Mustard-Treated Spleen Virus Injected 24 Hours after Preparation

Experiment M6

Pig no.	Results of injection			Results of challenge
	Symptoms		Lesions	
	Temp.	Visible		
M31	No	No	No	Immune
M32	No	No	No	Immune
M33	No	No	No	Immune
MC14(a)	Yes	Yes	Yes	-
MC15(b)	Yes	Yes	Yes	-

(a) Control for untreated virus.

(b) Control for challenge virus.

TABLE 13

Effect on Pigs of Mustard-Treated Spleen Virus

Experiment M7

Pig no.	Results of injection			Results of challenge (a)
	Symptoms		Lesions	
	Temp.	Visible		
M34	No	No	No	Immune
M35	No	No	No	Immune
M36	No	No	No	Immune
M37	No	No	No	Immune
M38	No	No	No	Immune
MC16(b)	Yes	Yes	Yes	-
MC17(c)	Yes	Yes	Yes	-
MC18(c)	Yes	Yes	Yes	-
MC19(d)	Yes	Yes	Yes	-

(a) Challenged by pen exposure.

(b) Controls for non-treated virus.

(c) Injected with virulent virus for purpose of pen exposure.

(d) Control to check spread of infection from MC17 and MC18.

TABLE 14

Summary of Effects on Pigs of Mustard-Treated Virus

Experiments M1 through M7

Number of Pigs	Type of virus	Hog cholera cases from virus	Number challenged	Immune to challenge
6	Blood	4	2	0
27[a]	Spleen	0	19[b]	19
5	Spleen	0	5[c]	5
10[d]	Untreated	10	0	-

(a) Eight died from causes other than hog cholera.

(b) Challenged by intramuscular injection of virulent virus.

(c) Challenged by pen exposure to sick pigs.

(d) Controls for untreated virus.

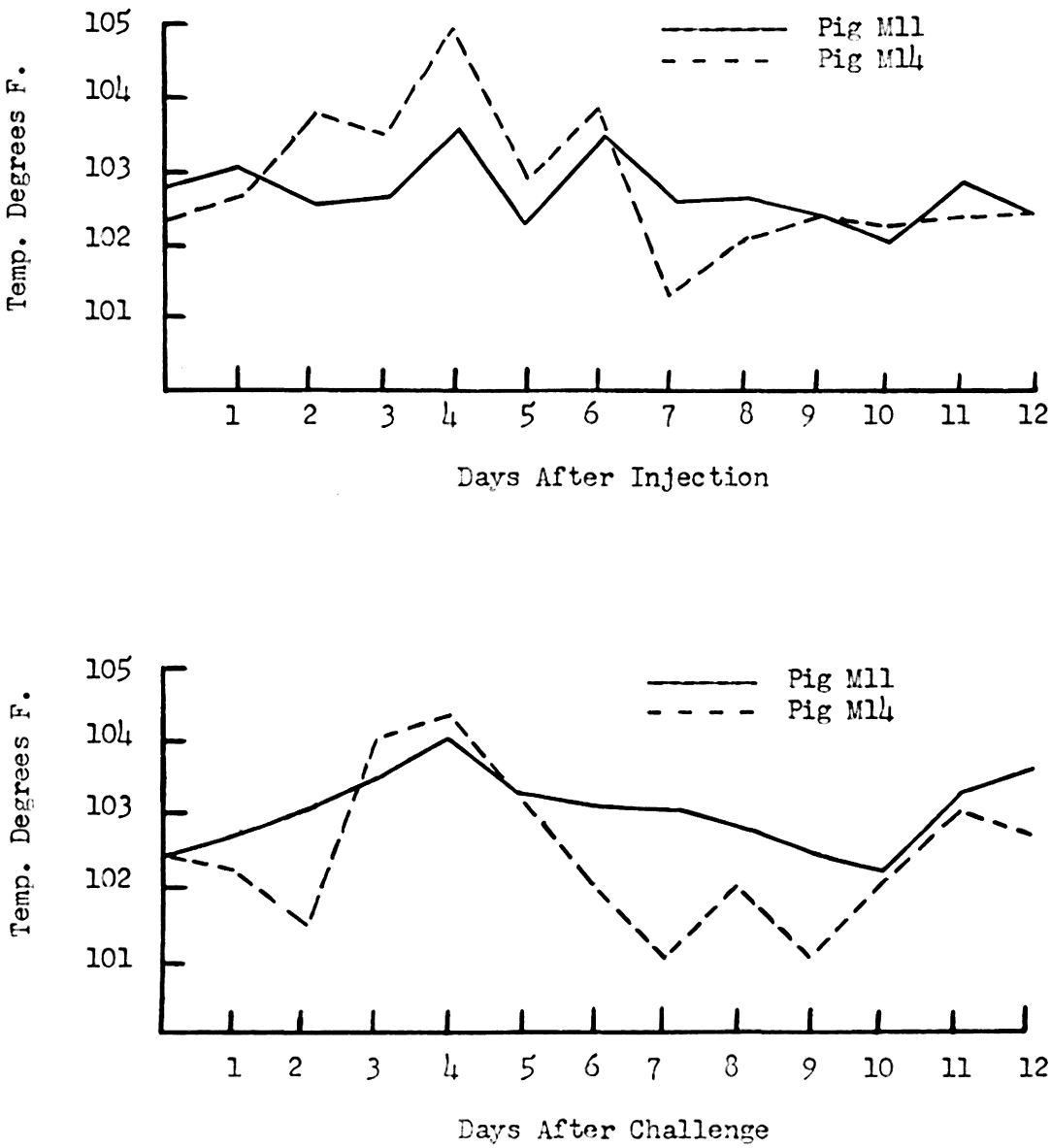

Figure 11. Temperature curves of pigs M11 and M14 after injection with mustard pig-spleen virus and after challenge with virulent virus.

DISCUSSION

Rabbit Passage of the Hog Cholera Virus

The hog cholera virus was passed alternately from pig to rabbit for six alternate passages and then carried for five serial passages in rabbits, as shown by Figure 5. Each pig injected with the rabbit-spleen virus prepared from the rabbits used in the alternate passages developed typical symptoms and on autopsy presented typical lesions of hog cholera. It is evident that the virus underwent no modifications during the alternate passages, since there was no reduction in virulence for the pig. The incubation periods, severity of symptoms, and extent of lesions were in agreement with those described by Hagan and Bruner (31), Udall (58), and Birch (7) as typical of hog cholera.

The control pig injected with rabbit-spleen virus at each serial passage, except the one injected with the sixth serial passage rabbit-spleen virus, developed typical symptoms and lesions of hog cholera (Figure 5). There was no evidence during the first five serial passages of modification in virulence of the virus for the pig, as determined by incubation times, severity of symptoms, and extent of lesions. The development of the typical disease in the control pigs indicated that the virus was present in the rabbit-spleen suspensions used for the first through the fifth serial passages.

The control pig injected with the rabbit-spleen virus prepared from the sixth rabbit in series remained normal. Challenge of this pig resulted in the development of the disease, indicating that immunization had not been accomplished. A second control pig responded in

the same manner. The control pig injected with the rabbit-spleen virus prepared from the seventh rabbit in series responded in just the same manner as the previous control. Repeated attempts to pass the virus from the fifth to the sixth rabbit in series were unsuccessful, as measured by the development of either the disease or immunity in the control pigs.

It is impossible to conclude from the data obtained that the hog cholera virus was modified by alternate passage from pig to rabbit for six such passages, followed by five serial passages in rabbits. Since all pigs used in the alternate passages and all control pigs used in the first five serial passages developed typical hog cholera, there was evidently no reduction in virulence of the virus up to this point. The first evidence of attenuation of the virus was obtained in the attempt to pass the virus from the fifth to the sixth rabbit in series (Figure 5). The failure of the sixth serial passage control pig (pig 12, Figure 5) to develop the disease seemed to indicate a loss of virulence by the virus. The susceptibility of the control pig (pig 12, Figure 5) to the challenge virus demonstrated the lack of immunizing potency of the sixth serial passage rabbit-spleen virus. If a modification of the virus had occurred, it had resulted in a change of the virus to a non-pathogenic and non-antigenic form. The absence of pathogenic or antigenic properties made it impossible to detect the virus after it had been carried for six alternate passages between the pig and rabbit and then for five serial passages in the rabbit; therefore, the question of modification or attenuation cannot be answered. Assuming that modification took place, it was not of a type that could

be put into practical uses, because of the lack of antigenic properties in the virus.

It is entirely possible and admittedly quite likely that no modification took place. The extreme virulence of the virus, as shown by Cole, Henley, and Hubbard (15), who found that the injection of as little as 1/2,500,000 cc. of blood virus was capable of infecting the pig, would support the view that the virus was carried unchanged from pig to rabbit and then serially through the rabbit. In this manner, successive dilutions of the virus could have been made, with the end-point appearing in the sixth serial passage (Figure 5).

No evidence was obtained that the virus became virulent for the rabbit. The rabbits did not present any symptoms of illness, nor did any develop lesions that might have been related to the injection of the hog cholera virus. The temperatures, taken at frequent intervals, as shown in Figure 2, did not reveal any febrile response, except in two instances when the rise in temperature occurred within four hours after the inoculation.

The development of hog cholera in the pigs used in the alternate passages and in the control pigs used in the serial passages indicates the survival of the hog cholera virus in the rabbits for at least 72 hours. Additional data on survival time of the virus in rabbits are shown in Figure 8. A susceptible pig injected with blood withdrawn from rabbit 5a at the end of 72 hours developed hog cholera, but the susceptible pig injected with blood drawn from rabbit 5a on the fourteenth day remained healthy until challenged. The virus survived for at least three days, but not for 14 days, in the rabbit. These data are

in agreement with that reported by Baker (4), Craig (18), and Koprowski, et al (42).

The results obtained in this study differ in several respects from those described by Baker (4) and Koprowski, James, and Cox (42). In this study, the hog cholera virus was either non-adaptable to the rabbit and was eventually lost through serial transfers or was completely adaptable, with no evidence of pathogenicity for the rabbit and a complete loss of pathogenicity and immunizing potency for the pig. Baker (4) was able to carry his strain A in serial passage in rabbits, which resulted in attenuation of the virus for the pig. The attenuated strain fully immunized swine to the virulent hog cholera virus. The strain B used by Baker did not become completely attenuated for swine and could not be carried beyond two serial transfers in rabbits. No evidence of pathogenicity for the rabbit by either strain A or B was obtained. Koprowski, James, and Cox (42) were able to carry the hog cholera virus, a Lederle strain, through rabbits, but failed to get complete attenuation of the virus for the pig. They obtained a febrile response in the rabbit as the only evidence of adaptation of the virus to the rabbit.

A satisfactory explanation of the varying degrees of adaptability to the rabbit of the various lots of virus used cannot be made. Since the techniques employed were essentially the same, this factor can be disregarded. The rabbits used came from different sources, but it is unlikely that the source of rabbits was responsible for the varying results. The most logical explanation is that, although the four lots of virus initially appeared to be identical, they were actually four

distinct and separate strains. Baker's strain A had been kept under laboratory conditions for a long period and had been cultured outside the body of the swine, after which it had been lyophilized and stored from 1941 to 1945, when it was injected into a pig. The spleen of this pig was kept frozen with dry ice until 1946. The fact that strain A had been handled in the manner just described may have influenced its adaptability to the rabbit, or, as Baker (4) states, it may have been naturally more adaptable. The strain B used by Baker was relatively recently isolated, which may account for its poor adaptability. The Lederle strain used by Koprowski, James, and Cox would also be considered a recent isolation, since it had been maintained in pigs. In effect, strain B (4), the Lederle strain (42), and the stock strain used in this study were similar in history and results, except in two major respects. First, the Lederle strain produced a febrile response in rabbits, whereas strain B (Baker) and the stock virus used for this work did not. Second, the stock virus used for this study was either completely inadaptable to the rabbit, or completely altered by passage through the rabbit, in contrast to the incomplete adaptation to the rabbit shown by the other two strains.

It appears that four distinct strains of the virus were used: strains A and B by Baker (4), Lederle strain by Koprowski, James, and Cox (42), and the stock strain used in the work reported here. It is apparent that the strain A used by Baker possessed attributes, not present in the other strains studied, that made it possible to adapt this strain to rabbits, with resulting attenuation for swine.

In spite of the volume of work done on hog cholera, well-defined strains of the virus have not been established. Many of the so-called "breaks" in the serum-virus method of immunization may conceivably be due to immunologically distinct strains or types, or even variants within types. The recent report of Dale, Schoening, Cole, Henley, and Zinober (19) confirms the existence of a variant and the importance of the variant in immunization "breaks". These workers, in studying losses in swine in 1949 and 1950, found that some of the virus recently used in the simultaneous method possessed unusual characteristics; this virus they designated as variant A. They found that pigs successfully immunized were resistant to the variant virus; however, susceptible pigs injected simultaneously with variant virus and with serum developed hog cholera. Similar pigs injected with standard virus and the same serum remained well.

Attempts to adapt the hog cholera virus to rabbits, which gave results indicating the differentiation of four strains, is additional evidence of the existence of distinct strains of the virus. It seems reasonable to assume that the rabbit passage technique offers a means of clearly differentiating strains of the hog cholera virus.

Ultraviolet Irradiation of the Hog Cholera Virus

The study of the effects of irradiation of the hog cholera virus, in the form of a spleen suspension, was done in a series of experiments.

The results of the first experiment, as seen in Table 1, suggested that the virus could be attenuated by irradiation for 60 minutes with the Westinghouse lamp CH4. Pig UV2 injected with the pig-spleen virus irradiated for 60 minutes developed no symptoms and was immune when

challenged. It is entirely possible, however, that this pig possessed an innate immunity to the virus and therefore developed no symptoms when injected with either irradiated pig-spleen virus or with virulent virus.

In the remaining experiments the WL793 lamp was used. Irradiation of the pig-spleen virus for 15 and 30 minutes by this lamp failed to attenuate the virus, as Table 2 shows. All pigs injected in these experiments developed the typical disease with no evidence of attenuation of the virus.

Irradiation of pig-spleen virus for 60 minutes with lamp WL793 failed to attenuate the virus for the pig. Examination of Tables 3 through 8 reveals the fact that the virus was not modified in virulence under the conditions of the experiments. Only one pig, UV19, (Table 4) failed to develop typical symptoms of hog cholera and was found to be immune on challenge. Two other pigs in the same experiment, UV18 and UV20, (Table 4) developed typical symptoms but recovered rapidly. They were challenged and found to be immune, a state that almost always exists in pigs that have recovered from the disease. The pigs in Experiment UV4 were litter mates and may possibly have had some inborn resistance to the virus, which might account for the slight difference in their response to the irradiated virus.

No evidence of partial attenuation of the virus was obtained, since incubation periods, severity of symptoms, and extent of lesions were in general similar to those seen in the controls. The summary, Table 6, reveals that only two pigs inoculated with irradiated virus failed to develop symptoms and were found immune when challenged, and that two

others developed symptoms but recovered.

The reason for the failure of irradiation of the pig-spleen virus to alter its infectivity for the pig is not evident from the data obtained. Hodes, Webster, and Lavin (35) found that rabies virus not cleared by sedimentation and centrifugation was not destroyed by an exposure of 60 minutes. The pig-spleen virus suspensions used in this study were allowed to sediment, after which the supernatant fluid was filtered through gauze and then further clarified by centrifugation. This procedure furnished a pig-spleen virus suspension that was free of any visible particles that might have protected the virus from the irradiation. The extremely small size of the hog cholera virus particles (31), however, may allow masking or protection by tissue particles of microscopic size.

Additional factors, according to Hodes, Webster, and Lavin (35), that influence the actinn of ultraviolet light are turbidity of solution, amount of shaking, kind of lamp, and presence of extraneous organic material. The pig-spleen virus used was turbid, but was not completely opaque, and it is not likely that turbidity was a major factor in the failure to attenuate the virus. The suspensions were constantly agitated during the exposure time, and, since the depth of the fluid was only one to two millimeters, inadequate agitation cannot be considered a factor.

The most plausible explanations for failure of the irradiation to attenuate the virus, therefore, appear to be either the kind of lamp, the presence of extraneous organic material, or the size of the virus. The lamps used were not strong sources of ultraviolet radiations, but, on the other hand, the duration of exposure was sufficiently long to allow adequate irradiation of the virus. Attenuation of the hog cholera

virus by ultraviolet irradiation should be reinvestigated when the techniques have been developed by which it will be possible to obtain concentrations of virus in suspensions containing little or no extraneous organic matter.

Nitrogen Mustard Treatment of the Virus

The study of the effects of nitrogen mustard (methyl-bis (β-chloroethyl)-amine) on the virulence and antigenic properties of the hog cholera virus was conducted in a series of experiments. Experiment M1 (Table 7), in which two lots of blood virus were used, did not give consistent results. The virus was destroyed, and neither symptoms nor immunity were shown by the pigs injected with mustard blood virus prepared from lot 1 blood virus. The virus was not attenuated, and typical hog cholera was produced in the pigs injected with the mustard blood virus prepared from lot 2 blood virus. It appears, therefore, that the effect of mustard on the virus, in the form of blood virus, probably depends upon the proportion of nitrogen mustard to virus. Since the amounts of nitrogen mustard used with each lot of blood virus were the same, it can be assumed that the concentrations of the virus in the two lots of blood were different. Johnson (39) stated that his studies with the fowl leucosis virus gave similar results, and that whether the virus was destroyed depended upon the amount of nitrogen mustard in relation to the concentration of virus. It is also interesting to note that Johnson (38) obtained evidence of a lethal effect of nitrogen mustard on the fowl leucosis virus when blood drawn from treated birds failed to transmit the disease.

The results obtained with the mustard pig-spleen virus used in

Experiments M2 through M7 were consistent. As Tables 8 through 13 demonstrate, the mustard pig-spleen virus did not produce visible symptoms in 32 susceptible pigs. Seven pigs developed a rise in temperature following the injection of the mustard pig-spleen virus. Of these seven, pig M14 (Table 9) showed a fever on the fifth day. A rise in temperature at this time, according to Hagan and Bruner (31), occurs after the injection of hog cholera virus into healthy, susceptible pigs. It must be assumed, therefore, that the febrile response in this pig was due to the hog cholera virus. The absence of a fever in the other pigs in the same experiment may indicate an unusual susceptibility of this particular pig. Six of the seven pigs developing a post-injection rise in temperature showed the rise on the twelfth day or later (Table 10). The development of a temperature this long after injection of the virus is not in agreement with the accepted febrile response of susceptible pigs to the virus. Four of six pigs died with secondary complications of liver damage from ascarid infection and verminous pneumonia. The hog cholera virus could not be demonstrated in tissues removed from the four pigs, and it can be concluded that the rise in temperature on or after the twelfth day in the six pigs in Experiment M4 (Table 10) was due to secondary causes and not to the hog cholera virus.

Eight of the 32 pigs injected with mustard pig-spleen virus died before challenge. In addition to the four pigs of Experiment M4 (Table 10) already noted, two others in the same group died from the same causes. With the exception of this one group of pigs, which apparently were not good experimental subjects, the losses of pigs were limited to one each in Experiments M2 and M3. In no instance could the hog cholera

virus be demonstrated in the pigs dying after the injection of mustard pig-spleen virus, and in all cases there were adequate secondary complications to account for the deaths. It can be concluded, therefore, that no deaths occurred as the result of injection of the mustard pig-spleen virus. It is also evident that mustard treatment of the pig-spleen virus reduced its virulence so that no ill effects resulted from the injection of mustard pig-spleen virus into susceptible pigs.

The mustard pig-spleen virus possessed an excellent immunizing potency. As shown by Tables 7 through 11 and by Table 14, 19 pigs were challenged with two milliliters of virulent virus three to four weeks after they had received five milliliters of mustard pig-spleen virus. None of the 19 pigs showed any visible symptoms of cholera following the challenge. Four of the 19 pigs challenged by the intramuscular injection of virulent virus developed a slight temperature rise on the third or fourth day after challenge. This temperature rise, according to Hagan and Bruner (31), is typical of the response of the healthy pig to the injection of the hog cholera virus and is similar to that seen in the control pigs. The absence of any visible symptoms and a transitory febrile response in only four pigs is evidence that the immunity in the 19 pigs challenged by the intramuscular injection of virulent virus was entirely satisfactory. The intramuscular injection of the hog cholera virus is the severest form of exposure used in studies of this disease. The degree of resistance shown by the pigs that had received the mustard pig-spleen virus to this form of challenge indicates that the mustard pig-spleen virus would adequately protect against the less severe field exposure.

Five pigs injected with mustard pig-spleen virus were challenged by pen exposure to sick pigs (Table 13). None of these five pigs developed symptoms of hog cholera, although susceptible pigs in the same pen contracted the disease from the cholera-infected pigs. The immunity in the five pigs was adequate against pen exposure, which was comparable to exposure in field outbreaks of the disease. This constitutes additional evidence that the mustard pig-spleen virus would adequately protect against field exposure.

No evidence was obtained that the mustard pig-spleen virus was spread from the injected pigs to non-injected pigs. In Experiment M7 (Table 13), three non-injected pigs were kept in contact with five pigs injected with mustard pig-spleen virus. The three pigs developed no symptoms of hog cholera as the result of the contact. The non-injected pigs later proved susceptible to virulent virus. Although the data are not extensive, it indicates that the mustard pig-spleen virus is not capable of passing from injected to non-injected pigs. It is evident that the mustard pig-spleen virus can be used in herds in which all susceptible pigs cannot be injected at the same time.

That it is not necessary to inject the mustard pig-spleen virus at the end of one hour after mixing the nitrogen mustard with the pig-spleen virus is shown in Table 12. Three pigs were injected with mustard pig-spleen virus 24 hours after the pig-spleen virus had been mixed with the nitrogen mustard. All three pigs exhibited satisfactory immunity when challenged three to four weeks after injection of the mustard pig-spleen virus. Two of the pigs, however, showed a slight transitory rise in temperature following the challenge dose of virus. Although the data

are not extensive, it appears that it is not necessary to inject the mustard pig-spleen virus immediately after its preparation; this allows the flexibility needed for field use.

The data obtained confirm the findings of Tenbroeck and Herriott (55), who demonstrated some immunity in pigs treated with mustard-treated hog cholera virus. The results obtained in this study differ in several respects from those of Tenbroeck and Herriott. They lost three pigs from acute hog cholera, whereas no pigs were lost from this cause in this study. All pigs challenged by these workers showed a rise in temperature following exposure to the virus by contact with infected pigs. Only four pigs in this study showed such a febrile response, and that followed a much more severe exposure (injection of the virulent virus) than was used by Tenbroeck and Herriott. In addition, data have been obtained showing that the pig injected with mustard-treated virus is not a source of infection for the non-injected, susceptible pig. Furthermore, limited data indicate that the mustard-treated virus need not be injected immediately after its preparation.

The demonstration that it is possible to attenuate the hog cholera virus by a nitrogen mustard and that the attenuated virus is highly antigenic gives investigators another method that can be used in the preparation of a hog cholera vaccine. The results obtained in this study lead logically to additional investigations. The optimum proportion of nitrogen mustard to virus for the preparation of a vaccine, the minimum dose of vaccine necessary for immunization, the best route of injection, and the duration of the immunity are among the problems to be investigated.

CONCLUSIONS

1. The adaptation of the hog cholera virus to the rabbit, with an attenuation in virulence for the pig without loss of antigenic potency, depends upon the particular strain of virus employed. The failure of the hog cholera virus used in this study to become adapted to the rabbit, with the varying degrees of adaptability demonstrated by others with virus stocks from various sources, gives evidence of the existence of strains distinguishable by means of their responses to passage in rabbits. It appears that the rabbit can be used as an experimental animal for the differentiation of strains of the virus.

2. The failure to attenuate the virus by the methods used shows that the virus is not readily susceptible to ultraviolet irradiation. Attenuation of the hog cholera virus by ultraviolet irradiation will depend upon either the use of a stronger source of ultraviolet radiations or a virus preparation relatively free of extraneous material.

3. The nitrogen mustard, methyl-bis (β-chloroethyl)-amine, attenuates the hog cholera virus when the virus is used in the form of a pig-spleen suspension. The virus is attenuated without a loss of antigenic potency, and the mustard-treated virus can be used for the successful immunization of pigs against the virulent form of the virus. The mustard-treated virus does not produce ill effects in the injected pigs, does not spread from injected pigs to non-injected pigs, and, furthermore, it can be used for immunizing pigs for at least 24 hours after its preparation. The hog cholera virus attenuated by nitrogen mustard may have wide application as an immunizing agent.

SUMMARY

Rabbit Passage of the Virus

The hog cholera virus was carried through six alternate passages from pig to rabbit and then for five serial passages in rabbits. The virus could not be demonstrated in rabbit-spleen suspension prepared for the sixth serial passage in rabbits. The lack of pathogenic or antigenic properties for the pig made it impossible to detect the virus, assuming that it was present in the rabbit-spleen suspension, at the sixth serial passage. The virus did not become pathogenic for the rabbit during the alternate and serial passages, although it survived for at least 72 hours in the rabbit. No evidence of attenuation of the virus for the pig was obtained in either the alternate or serial passages.

Ultraviolet Irradiation of the Virus

The hog cholera virus in the form of a pig-spleen suspension was irradiated by the Westinghouse lamps CH4 and WL793. The virus was irradiated at a distance of 15 centimeters from the lamp. The suspension was placed in an open petri dish, to a depth of 1 to 2 millimeters, and constantly agitated during the irradiation. Irradiation by the CH4 lamp for 30 and 60 minutes and irradiation by the WL793 lamp for 15, 30, and 60 minutes failed to attenuate the virus for the pig. Typical hog cholera was produced in pigs injected with the irradiated virus.

Nitrogen Mustard Treatment of the Virus

The hog cholera virus in the form of blood virus was mixed with

the nitrogen mustard, methyl-bis (β-chloroethyl)-amine, in the proportion of five milliliters of blood virus to ten milligrams of nitrogen mustard. In one lot of blood virus so treated, the virus was completely destroyed, whereas in a second lot the virus was not affected.

Treatment of five milliliters of the virus, in the form of pig-spleen suspension, with ten milligrams of the nitrogen mustard completely attenuated the virus for the pig, so that, when the virus was injected into pigs, no ill effects appeared. Pigs injected with five milliliters of the mustard-treated pig-spleen virus developed a resistance to the virulent virus. The injected pigs successfully withstood the intramuscular injection of two milliliters of virulent virus, or pen exposure to cholera-infected pigs. The mustard-treated pig-spleen virus produced a satisfactory immunity in pigs injected with the treated virus 24 hours after its preparation. The mustard-treated pig-spleen virus did not spread from injected pigs to non-injected pigs kept in close contact with them.

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