AN ATTEMPT TO PROPAGATE THE MOUSE-ADAPTED LANSING STRAIN OF POLIOMYELITIS VIRUS IN THE DEVELOPING CHICKEN EMBRYO

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

Lenore Jones

1949

This is to certify that the

thesis entitled

An Attempt to Propagate the
Mouse-Adapted Lansing Strain
of Poliomyelitis Virus in the
Developing Chicken Embryo

presented by

Lenore Jones

has been accepted towards fulfillment
of the requirements for

Master's degree in Bacteriology

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Date November 10, 1949

AN ATTEMPT TO PROPAGATE THE MOUSE-ADAPTED LANSING STRAIN OF POLIOMYELITIS VIRUS IN THE DEVELOPING CHICKEN EMBRYO

BY

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A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Bacteriology and Public Health

1949

ACKNOWLEDGMENT

The author wishes to express sincere appreciation to the Michigan Department of Health, to Dr. G. D. Cummings, Director, Division of Laboratories, for the privilege of carrying out this study in the Virology Section; to Dr. Serge G. Lensen, Acting Chief, Section of Virology, for his example as a teacher and his valuable supervision and advice in this work; and to the personnel of the Section of Virology for their cooperation.

The author also wishes to express appreciation to Dr. H. J. Stafseth, Professor and Head of the Department of Bacteriology, Michigan State College, for his helpful suggestions and criticisms of this thesis.

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INTRODUCTION

Poliomyelitis is a common virus disease of man which presents, in clinically typical cases, flaccid paralysis of groups of voluntary muscles, resulting from the destruction of motor neurons in the brain and spinal cord. Most cases, however, run only a mild course characterized by fever, headache and upper respiratory and gastro-intestinal symptoms.

Most poliomyelitis strains can be transmitted only to monkeys and chimpanzees. A few strains of poliomyelitis virus, the Lansing, the Yale - SK, MEF1, Phillips, WW and Wfd strains are pathogenic for mice, cotton rats and hamsters as well as primates. Several strains of poliomyelitis virus have been grown in tissue cultures but as yet there are no substantiated reports of the growth of strains pathogenic for monkeys in developing chicken embryos.

At the present time, diagnostic tests are limited to the isolation of the virus and its identification by the clinical manifestations and histopathologic findings of the disease reproduced in monkeys. The mouse neutralization test has been used, but this test is limited to the rodent-adapted strains.

In the event that developing chicken embryos could be used in the primary isolation of the poliomyelitis virus, or that rodent-adapted strains could be adapted to eggs, it would not only mean an increase in the host range but also a more practical, inexpensive method for experimental studies or diagnostic work. If the poliomyelitis virus could be propagated in the chicken embryo, the attempt might then be made to prepare a suitable complement-fixing antigen from egg material.

It is the purpose of this paper to report the methods and results of an attempt to propagate the mouse-adapted Lansing strain of poliomyelitis virus in developing chicken embryos.

I. GENERAL CONSIDERATIONS

A. Etiology of Poliomyelitis

The etiologic agent of human poliomyelitis is extremely small, its average diameter being estimated as 8-12 mu on the basis of filtration experiments with gradacol membranes (Elford et al, 1935)[1]. The virus is quite sensitive to oxidizing agents, ultraviolet light and heat, being inactivated by the latter within three minutes at a temperature of 50°C, (Shaughnessy, 1930)[2]. Inactivation of the virus has been shown to occur with 0.05 p.p.m. of free chlorine at a pH 6.85 - 7.40 in ten minutes, (Lensen et al, 1947)[3], and with 0.1 gm per cent mercuric chloride at 37°C within two hours, (Schultz and Robinson 1942)[4]. It is relatively stable even at extremes of the pH range and is resistant to ether, merthiolate, penicillin and streptomycin. Brodie (1935)[5], found that it is inactivated in 3.5 days at icebox temperature by 0.3 per cent formalin, but resists 0.2 per cent formalin and 1 per cent phenol for 10 days.

Preservation of the virus may be carried out by storing infected entire central nervous system (CNS) in 50 per cent glycerine in the refrigerator. According to Melnick (1946)[6], the virus can be stored at -20°C as well as -70°C for twelve months without loss of titer. Virus suspensions, purified by ultracentrifugation and stored at a temperature of -25°C to -30°C will show a considerable drop in titer within a few days, (Lensen, unpublished observation).

B. Portal and Route of Invasion

Poliomyelitis virus produces histopathologic changes only in the CNS. It may be isolated, however, from the intestinal wall and the intestinal content as well as from the CNS of fatal cases. The virus may also be found in the feces and nasopharyngeal washings of patients and

asymptomatic carriers. It has never been found in the spinal fluid or lymphatics but has been recovered occasionally from the blood of patients, (Ward, 1946)[7] and (Koprowski, 1947)[8], as well as from the blood of monkeys infected by the intracerebral route, (Sabin, 1944)[9].

The portal and the route of invasion from the primary site of infection to the CNS has not been determined definitely. For a long time, it was believed that the virus entered, and passed from the nasopharynx by way of the olfactory nerves to the brain and spinal cord, but examination of the olfactory mucosa and olfactory bulbs, at necropsy of patients, has only occasionally revealed virus or evidence of invasion (Howe, 1948)[10].

Regions of the alimentary canal from which the virus has been isolated include the tongue, the walls of the oropharynx, the duodenum, ileum and colon (Sabin, 1947)[11]. Whether there is multiplication of the virus in the alimentary canal with the subsequent invasion and extension from there to the CNS, or whether this is merely the pathway of excretion, has been the subject of considerable investigation and controversy.

Chimpanzees have been infected with poliomyelitis by feeding them food which has been exposed to flies trapped in homes of patients ill with poliomyelitis, (Melnick, 1947)[12]. Experiments with the cynomolgous monkeys indicate that the virus may enter through the pharyngeal or intestinal walls and after multiplying locally, reach the CNS by way of the autonomic nerves, (Burnet and Jackson, 1940)[13]. Faber, Silverberg and Dong (1948)[14], also report infection of three of six monkeys (cynomolgous, Macaca irus)[15], after ingestion of food mixed with poliomyelitis virus. Microscopic examination showed that the route of infection was not olfactory, but did show, by the distribution of lesions in the peripheral ganglia and CNS, extension from the oropharyngeal mucosa in two monkeys, via the trigeminal and cervical sympathetic nerves; and in

the third monkey, from the intestinal mucosa via the intestinal sympathetic system to the CNS.

It has been found in human cases of poliomyelitis occurring within 60 days after tonsillectomy that there was a marked incidence of the bulbar form (Aycock, 1942)[16]. This would suggest that tonsillectomy plays a part in the inoculation of the exposed fibers of the glossopharyngeal nerve which can then be followed by the extension of the virus along the axons of the glassopharyngeal nerve to the nuclei of the medulla and brain.

Others claim that in human cases in which virus may be found in the intestine, it is merely an indication that this is the route of exretion of the virus. Melnick was able to isolate poliomyelitis virus from the stools of a monkey inoculated by the parenteral route, (Melnick, 1946)[17]. Howe (1948)[18] states, "It is unlikely that the virus propagates in the intestinal contents or that its invasion extends as far as the CNS." He sites the work of Sabin and Ward, (1941)[19] and Howe and Bodian, (1947)[20], who have shown that neither virus nor lesions have been found in the coeliac ganglin, but he mentions the possibility of migration of the virus from the gastro-intestinal tract along the vagus and visceral afferents to the CNS.

C. Histopathology

Lesions of poliomyelitis are highly characteristic in their nature and distribution in the brain and spinal cord wherever neurons have been destroyed by the virus. Neuron damage involves chromatolysis , neuronal necrosis, neuronophagia and "outfall" of cells. This is accompanied by an inflammatory reaction in the surrounding mesodermal tissue in which the area becomes infiltrated with lymphocytes, plasma cells and macrophages. Perivascular cuffing, (infiltration of adventitia by lymphocytes) is

nearly always present in the neighboring arterioles and venules.

In the spinal cord and medulla, lesions are found primarily in the grey matter of the anterior horn, and to a lesser extent in the autonomic and sensory columns. In the brain, the precentral gyrus of the cerebral cortex, the vermis and the deep cerebellar nuclei, and occasionally the basis portis and inferior olives may show typical pathological changes.

D. Immunologic Types of Poliomyelitis Viruses

Two groups of experimental workers, Howe, Bodian and Morgan, (1949)[21], and Kessel and Pait (1948)[22], have shown that there are at least three immunologically different groups or types of poliomyelitis viruses. Three methods were used by these investigators in their immunological studies, namely: (1), injection of monkeys with one strain and reinjection with a heterologous strain; (2), cross immunity experiments (immunization of monkeys with various strains, challenging them with the homologous strain and then rechallenging with a heterologous strain) and (3), neutralization tests in monkeys.

The three types described by Howe, Bodian and Morgan correspond with the three types described by Kessel and Pait wherever the same strains were used.

Each found the Type I or Brunhilde Type to include the following strains, Brunhilde, Kotter, Minneapolis and Frederick. Howe et al added Per, Riley, Sudeck, Beich and MEF2 strains to this group, while Kessel and Pait included strains designated as BK, McK, Cu, Gu and Cp in their classification.

Type II, or the Lansing Type, includes, according to Kessel and Pait, the Lansing and MV strains and according to Bodian (1949)[23], the rodent-adapted strains, Lansing, Yale - SK, MEF1, Phillips, Wfd and WW strains and the non-rodent-adapted strains, the MV, McC and Aycock strains.

Type III, or the Leon Type, was found by both groups of investigators to include only one strain, the Leon strain. This strain was shown to be unrelated to either the Lansing or Brunhilde Types.

E. Definition of the Lansing Type of Poliomyelitis Viruses

Poliomyelitis has been classified as a clinical entity since first described by Howe in 1840, but laboratory studies of the virus were not begun until 1909 when Lansteiner and Popper succeeded in transmitting the disease from a human cord suspension to the monkey.

The next important step in the development of experimental work in poliomyelitis was the adaptation of the Lansing strain of poliomyelitis virus to the white mouse, (Armstrong, 1939)[24]. This was achieved by transmitting the disease from a human cord suspension first to a monkey, then to cotton rats and subsequently to mice.

Since then other strains of the Lansing Type have been shown to be pathogenic for mice, namely, the MEFl, WW, Wfd, Yale - SK and Phillips strains.

These strains meet the criteria of a poliomyelitis virus as outlined by the Committee on Nomenclature of the National Foundation for Infantile Paralysis.

Criteria of a Poliomyelitis Virus

(1) The strain must have been described as the cause of human poliomyelitis.

(2) The rodent passage virus must be transmissible to primates.

(3) The histopathological lesions in the spinal cord, medulla or brain of the paralyzed monkey must be characteristic for poliomyelitis.

(4) The strain must be identified as a poliomyelitis virus by immunologic tests with monkeys.

(5) The host range of a poliomyelitis strain thus far is limited to primates with the exception of the Lansing Group which also are pathogenic for mice, cotton rats and hamsters. Any new virus which is atypical with respect to host range should be classified only after complete consideration of its other properties.

All attempts to cultivate true poliomyelitis strains in the developing chicken embryo have been unsuccessful.

(6) Another important property in identification of poliomyelitis virus is its small particle size (ranging from 8-12 mu).

Considerable confusion has arisen from the association of the rodent-adapted Lansing Type of poliomyelitis viruses with the so-called "murine poliomyelitis" viruses. These viruses include the strains producing spontaneous mouse encephalitis, (e.g., Theiler (TO, FA and GDVII strains), as well as the Columbia-SK, the MM and the C(M) Lansing strain, (Schultz and Enright, 1948)[25]. They produce paralysis or encephalomyelitic symptoms in mice but have not been shown to produce poliomyelitis in man. They have a wider host range than that of the Lansing Type and have been propagated in the embryonated chick egg.

Because of their wide host range, the so-called "murine poliomyelitis" viruses are much easier to use for experimental studies. Some investigators believe that they may provide a means of understanding better the true poliomyelitis viruses, especially if it could be shown that these viruses and the Lansing Type have a common evolutionary origin. On the other hand, the Committee on Nomenclature of the National Foundation for Infantile Paralysis believes that these murine neurotropic viruses have often been the source of confusion and error in work with poliomyelitis viruses in rodents.

The Theiler viruses are of interest in any experimental work with the Lansing Type in mice because of the possibility of picking up this spontaneous mouse encephalomyelitis virus.

The Theiler strains, the C(M) Lansing, Columbia-SK and MM strains are also of interest because they have been propagated in the

developing chicken embryo and many reports of the methods used in their adaptation to the embryonated egg have been published. Few reports have been published, however, on the methods used by the various investigators who have attempted to propagate true strains of poliomyelitis in the chick embryo.

II. THE USE OF CHICKEN EMBRYO TECHNIQUES IN VIROLOGY

Since viruses will grow only in living, susceptible cells, it has been a major problem in experimental virology to find animal hosts which may be obtained in sufficiently large numbers for virus cultivation. Many viruses have been propagated in the developing chicken egg and the working out of chicken embryo techniques has promoted the development of virology.

Goodpasture and Buddingh, and Burnet and Beveridge were among the first to recognize the potentialities of virus propagation in the chicken embryo. Their extensive research with vaccinia, fowlpox, Newcastle disease virus, etc., and their various publications on methods for virus propagation in the chicken embryo have been outstanding contributions to the development of diagnostic and experimental virology.

Most animal viruses have been propagated in the chicken embryo but a few, e.g., poliomyelitis, herpes zoster, dengue and trachoma, have not been propagated in eggs in spite of the efforts of many investigators.

Propagation of viruses in the chicken embryo has three practical medical uses: (1) Identification of the etiologic agent by direct isolation of the virus concerned, e.g., in the differential diagnosis of smallpox and chickenpox. (2) Preparation of antigens for serodiagnostic work, e.g., the Frei test in lymphogranuloma infection; complement fixation in psittacosis, equine encephalomyelitis and influenza; and hemagglutination inhibition tests for influenza. (3) Preparation of vaccines for prophylactic use against yellow fever, influenza, and Eastern and Western equine encephalomyelitis.

Different routes of inoculation are employed according to whether the chicken embryo is inoculated for primary isolation of a virus, for

vaccine or antigen preparation, or whether it is to be used for a pock-counting method.

Amniotic inoculation exposes the inner epithelial lining of the amnion and the epidermal epithelium of the chicken embryo to the virus. This method utilizes the respiratory and gastro-intestinal tracts as portals of entry. Chicken embryos thirteen days of age are employed for the primary isolation of influenza A strains because at this stage in the development of the chicken embryo, the epithelial cells of the trachea are most susceptible to the virus. On the other hand, the less specialized cells of the eight-day embryo seem to be more susceptible to influenza B, herpes simplex and vaccinia viruses.

Allantoic inoculation is particularly useful in the propagation of the influenza and psittacosis group viruses for preparation of complement-fixing antigens. This route is also used in the production of influenza vaccine and in preparation of antigen for the hemagglutination test.

Yolk sac inoculation is employed for the primary isolation of the viruses of lymphogranuloma venereum, psittacosis and mumps. The cells of the embryonic brain may be infected by intracerebral inoculation with the viruses of herpes, (Anderson, 1940)[26], and rabies, (Dawson, 1941)[27].

Several viruses such as those of variola, vaccinia, herpes simplex, fowlpox and influenza produce discrete opaque lesions called "pocks" when inoculated on the chorioallantoic membrane. Burnet and his co-workers (1946)[28a], have employed pock-counting methods for the titration of virus suspensions and serum-virus mixtures. Chorioallantoic inoculation may also be used for primary isolation of some viruses, e.g., louping ill and St. Louis encephalitis. These viruses when placed on the chorioallantoic membrane will invade the body of the embryo.

III. PREVIOUS ATTEMPTS TO PROPAGATE POLIOMYELITIS VIRUS IN THE CHICKEN EMBRYO

Gard, in 1943, inoculated chicken embryos by the chorioallantoic route with a monkey cord suspension of the L strain of poliomyelitis virus. He reported a single infection of a monkey which had been inoculated intracerebrally with 0.5 ml. of a chicken embryo suspension. Since the chicken brain suspension was obtained from the first egg passage only, there is the possibility that reproduction of the disease in the monkey was due to the passive transfer of the virus.

All other workers were unable to propagate the virus of poliomyelitis in the developing embryo. Reports have been published by Burnet (1935)[28b], Stimpert (1939)[29]; Kast and Kolmer (1948)[30]; Riordan and Sa Fleitas (1946)[31]; Schultz and Enright (1948)[32]; and Jamieson and Powell (1948)[33b].

Methods Described by Burnet (1935)[28b]

Burnet attempted to propagate poliomyelitis viruses from five human cord suspensions and one monkey cord suspension (MV strain) in the chicken embryo. Ten per cent suspensions of spinal cords were prepared by grinding and emulsifying the infected material in broth, centrifuging and removing the supernatant fluid for egg inoculation. Ten-day chicken embryos were inoculated with 0.1 ml. of this suspension by the chorioallantoic route. Three to six days after the inoculation, chorioallantoic membranes and embryo brains were taken for the second egg passage.

No gross or microscopic lesions were observed in the chicken embryo. Monkeys were inoculated with suspension from the first and second egg passages but none developed paralysis.

Methods Described by Riordan and Sa Fleitas

Riordan and Sa Fleitas (1946)[31] reported that their efforts to propagate in the chicken embryo the Lansing, Yale - SK, and Phillips strain, (mouse passage virus), as well as two strains of poliomyelitis virus from a monkey cord, and a strain from a human spinal cord, were unsuccessful.

Both the chorioallantoic and intracerebral routes were chosen for chicken embryo inoculation. For the chorioallantoic route, six-day embryos were inoculated with 10 per cent and 20 per cent suspensions of Lansing, Yale - SK and Phillips strains and the human and monkey cord suspensions. For the intracerebral route passages, ten-day chicken embryos were inoculated with 20 per cent suspensions. The eggs were harvested daily or at regular intervals ranging from four to thirteen days after inoculation. Chorioallantoic membranes, brains, spinal cords and embryo minus the C.N.S. were harvested. Suspensions from the first egg passage were used for mouse and monkey infectivity tests.

The mice and monkeys were observed for a period of thirty-five days and all remained well.

Schultz and Enright (1948)[32] and Jamieson and Powell (1948)[33a] have reported that they have attempted to propagate the Lansing strain in embryonated chicken eggs without success, but neither group has published a description of the methods which they used.

IV. THE PROPAGATION OF THEILER VIRUS (TO AND FA STRAINS), THE COLUMBIA SK, C(M) LANSING AND MM VIRUSES IN CHICKEN EMBRYOS

Methods used in the Propagation of the Theiler Virus

Several strains of mouse encephalomyelitis virus have been propagated in the chicken embryo by a number of investigators, including Gard (1943); Riordan and Sa Fleitas (1946)[31]; Durham and Parker (1943), and Schultz and Enright (1948)[32].

Riordan and Sa Fleitas reported the cultivation of FA and TO strains of Theiler's mouse encephalomyelitis viruses. The FA strain was propagated through ten passages of the developing chicken embryo. It was found that embryos from 6-10 days of age were satisfactory for inoculation. The choricallantoic, yolk sac, allantoic, and intracerebral routes were used. The inoculum chosen was 0.1 ml. of a 20 per cent suspension of mouse cord and medulla except for the intracerebral route which was 0.03 ml. Ten per cent suspensions of choricallantoic membranes, yolk sacs or whole embryos were used for subsequent egg passages. The highest concentration was found to be in the embryo inoculated at 6-7 days of age and incubated for 12-14 days at 35°C.

Theiler's TO strain was passed through four generations in the chicken embryo following the inoculation by the chorioallantoic route. Virus was found to be present in the highest titer in the chorioallantoic membranes and chicken embryo after twelve days incubation at 35°C following inoculation of six-day old eggs.

The TO and FA strains did not undergo any change after egg passage. The symptoms in mice inoculated with infected egg suspensions were similar to those produced by mouse to mouse passage.

Cultivation in the Chicken Embryo of Columbia-SK, MM and C(M) Lansing Strains

Schultz and Enright (1947)[32] reported the successful propagation

of the Columbia SK, the MM and the C(M) Lansing strains in the chicken embryo. They found that the highest concentration of virus was obtained when chicken embryos ranging from 8-14 days were inoculated. The inoculum used for the first egg passage was 0.1 ml. of a 10^{-5} dilution of a mouse brain suspension. The dilution 10^{-5} was chosen because it induced infection in three of three mice inoculated with 0.025 ml. intracerebrally. Dilutions 10^{-6} and 10^{-7} did not produce infection in all the mice inoculated.

The routes of inoculation used were the chorioallantoic, the yolk sac and intraembryonic. Heads, chorioallantoic membranes and abdominal viscera were harvested, ground to a 10 per cent suspension, centrifuged at 1000 r.p.m. and filtered through a Mandler candle. One-tenth ml. of this suspension was used for the egg passage inoculum. Chicken embryos which died 2-5 days after inoculation were stored in the refrigerator. On the fifth day after inoculation, all the surviving chicken embryos were harvested, pooled with the dead embryos and suspensions were prepared for the next egg passage.

In all, thirty serial egg passages were carried through with the Columbia SK strain, ten passages with the MM strain and fifteen with the C(M) Lansing strain.

The incidence of death among embryos in eggs incubated at 37°C was forty per cent lower than in eggs incubated at 35°C. Neutralization tests in mice showed that the Columbia-SK and the C(M) Lansing strains were neutralized by their homologous antiserum. Neutralization tests were not reported for the MM strain.

V. TISSUE CULTURE METHODS

Gildemeister (1933)[34] claimed that poliomyelitis virus can be propagated through at least 18 tissue cultures using chicken embryo brain tissue, monkey serum and Tyrode suspensions. Carrel flasks were used. Subcultures were made twice weekly. Pauli (1934)[35] was able to confirm this but all other investigators, including Plotz (1938)[36], Sabin and Olitsky (1939)[37] and Kast and Kolmer (1937)[38] were unable to repeat it.

Sabin and Olitsky (1939)[37] successfully propagated the MV strain of monkey passage virus using fragments of embryonic brain, obtained for young human fetuses.

Enders, Wilber and Robbins (1949)[39] report the cultivation of the mouse adapted Lansing strain of poliomyelitis virus in human embryonic tissue from the arm and leg.

In this case, proliferation of the virus must have occurred in tissue which does not contain intact neurons, that is, either in the peripheral nerve processes or in cells of mesodermal origin.

VI. EXPERIMENTAL METHODS USED IN AN ATTEMPT TO PROPAGATE THE MOUSE ADAPTED LANSING STRAIN OF POLIOMYELITIS VIRUS IN DEVELOPING CHICKEN EMBRYOS

Attempts to propagate the mouse-adapted Lansing strain of poliomyelitis virus in embryonating chicken eggs were made using the method of serial egg passage. Three to five serial egg passages were carried out in each experiment. The routes of inoculation chosen included the amniotic and intracerebral. At least four chicken embryos were inoculated in each passage. Fertile hens' eggs were inoculated after incubation at 37°C for 7-11 days. They were then incubated at 35° or 37°C for 2-5 days before being harvested. To check for the presence of virus, material harvested from each egg passage was inoculated into at least five mice (0.03 ml. intracerebrally). See Table I.

A. Inoculum Used for the First Egg Passage

Mice were inoculated with the Lansing strain of poliomyelitis virus. Spinal cords and medullae were harvested from mice which became paralyzed 2-5 days after inoculation. In experiments 1 and 2, one per cent suspensions of spinal cords and medullae were used, and in all the other experiments, ten per cent suspensions were used for the first egg passage inoculation. Whenever possible, eggs were inoculated the same day on which the mouse passage virus was harvested. Otherwise, the mouse cords and medullae were stored at -30°c.

Mouse passage virus preparations AM14K1M41, AM14K1M46, AM14K1M47 and AM14K1M48 were used. "A" designates the virus as it was received several years ago by the Section of Virology, Michigan Department of Health from Dr. Charles Armstrong. "M14" stands for the number of monkey passages carried out and "K" stands for one cotton rat passage. The second "M" designates the number of mouse passages following the cotton rat passage.

Titrations were carried out to determine whether a pool of amniotic fluids from normal 10-day chicken embryos could inactivate the Lansing virus and also to determine whether in inoculating embryos by the amniotic route the virus would be diluted beyond a titer which would produce paralysis in mice. Virus suspensions were titrated using (1) physiological saline and (2), amniotic fluids from normal 10-day chicken embryos as diluents. The dilutions were made and incubated for three days at 35°C in sealed test tubes to carry out as nearly as possible the conditions of incubation of the virus which prevail in the amniotic sac.

A titration of the virus suspension diluted with penicillin solution (1000 units per ml. inoculum), and streptomycin (8000 units per ml. inoculum), was carried out to determine whether these antibiotics would inactivate the virus. In some experiments, 25000 units of streptomycin was used for egg passage inoculation, but 8000 units per ml. of inoculum was the largest dose that mice could be given without developing convulsions. As before, dilutions were incubated three days at 35°C in sealed test tubes before the mice were inoculated.

There was no appreciable difference in the titers. The LD_{50} titer of the virus suspension diluted with amniotic fluids was .02 per cent. A normal 10-day chicken embryo will yield approximately 3.0 ml. of amniotic fluid, and if 0.1 ml. of 10 per cent virus suspension is inoculated into the amniotic sac, the virus would be diluted to 0.3 per cent. Since this dilution is considerably lower than that of the LD_{50} obtained in the titration, recovery of the virus after the first egg passage would be due probably to passive transfer rather than proliferation of the virus.

B. Experiments in which the Amniotic Sac Inoculation was Used

Nine experiments were carried out in which the amniotic sac was

chosen for the route of inoculation, (see Table I). The size of the inoculum ranged from 0.03 ml. in experiments 1 and 3; 0.1 ml. in experiments 4 - 8 to 0.25 ml. in experiment 9.

Technique of Amniotic Sac Inoculation

The eggs are candled and opened over the air sac. A drop of saline is placed on the shell membrane, then the membrane is ruptured by placing the inoculating needle at an angle of 45° below the drop and applying pressue so that a small hole is made through which the drop of saline will pass to make a liquid wedge between the shell membrane and chorioallantoic membrane. A portion of the shell membrane is then removed very carefully to give complete visibility of the route for amniotic sac inoculation. Gauge 22 or 23 needles, 5/8 inch in length, are used. The amniotic cavity is entered by introducing the needle through the allantoic sac as near the edge of the yolk sac as possible without rupturing it. The weight of the yolk will then serve to pull the amnion over the needle as it is pierced by a short, sharp thrust of the needle. The inoculated eggs are sealed with scotch tape and incubated at 35° or 37°C.

The collection of the amniotic fluids is carried out by cutting away the scotch tape over the air sac, rupturing the allantoic sac and then very carefully dropping the entire contents of the egg into a sterile petri dish without breaking the amnion. The amniotic fluid may then be drawn off with a needle and syringe.

Material Harvested from Eggs and the Preparation of this Material for the Next Egg Passage

Harvests consisted of (1), amniotic fluids pooled with brain and spinal column; (2), amniotic fluids pooled with heads and torsos; (3),

amniotic fluid; (4), brain and spinal column and (5), heads and torsos.

In experiments 1 - 4, brains and spinal columns or heads and torsos were ground to a 10 per cent or a 20 per cent suspension with physiological saline. In experiments 5 - 8, amniotic fluids were pooled with the embryonic tissues and ground to a 10 per cent or 20 per cent suspension with the physiological saline. These suspensions were centrifuged at 3000 r.p.m. for 15 minutes and the supernatant fluids were used for the next egg inoculation and for mouse inoculation.

Sterility Tests

Blood plates and N.I.H. thioglycolate sterility broth were inoculated with each chicken embryo suspension and mouse passage virus suspension before the suspensions were used as inocula for eggs or mice. Only sterile suspensions were used for the inoculation of the first and second egg passages. If bacterial contamination occurred in suspensions for egg passages 3 - 5, they were treated with antibiotics, (1000 units penicillin and 25000 units of streptomycin per ml. of inoculum, with a contact period of 30 minutes before the inoculation). When mice were inoculated, no more than 8000 units were used because a greater number of units cause convulsions.

Selection of Embryonated Egg Material for Serial Egg Passages

Embryos dying within 24 hours after the inoculation were discarded. Suspensions prepared from the chicken embryos dying 2 - 5 days after inoculation in the first two egg passages were passed separately to the next egg passage and mice were inoculated. The surviving embryos were harvested after 5 days and pooled for the next egg passage and mouse inoculation.

In passages 3 - 5, chicken embryos which died 2 - 5 days after inoculation were harvested and stored in the refrigerator. On the fifth

day after egg inoculation, all the surviving embryos were harvested, pooled with the dead embryos and suspensions were prepared for the next egg passage. Any suspensions showing bacterial contamination were treated with antibiotics as described under "Sterility Tests", before being used for egg or mouse inoculation.

Results of Mouse Inoculation: Experiments 1 - 9 in which the Amniotic Route of Inoculation was Used

Evidence of virus was obtained from chicken embryo suspensions which were harvested only from the first egg passages in experiments 3, 4, 5 and 8 when mice were inoculated. No mice became paralyzed after inoculation with suspensions from egg passage 2 - 5 in any of the experiments. In all cases in which paralysis of the mice occurred, the paralysis was typical of that produced by the Lansing strain.

In experiment 9, however, where AM14K1M48 passage stock virus was used, atypical symptoms in mice were produced when they were injected with sterile chicken embryo suspensions from egg passage 1 - 5. These mice developed encephalitic symptoms, tremors, ruffled fur and weakness rather than paralysis of the legs and toes. This experiment will be described later in this report.

Description of the Experiments in which the Lansing Strain Virus was Recovered from Chick Embryo Suspensions

Experiment 3

The inoculum chosen for the first egg passage was 0.03 ml. of a 10 per cent suspension of mouse passage virus. Seven-day chicken embryos were inoculated by the amniotic sac route. Heads and torsos were harvested, ground to a 20 per cent suspension, centrifuged, and eggs and mice were inoculated on the same day with a portion of this supernatant fluid. The remainder of the suspension was concentrated seven times, approximately, by ultracentrifugation for the next egg passage and mouse inoculation.

One of ten mice inoculated with the 20 per cent suspension became paralyzed 18 days after the inoculation. One of ten mice inoculated with the concentrated suspension of chicken embryo tissue became paralyzed after twenty days. None of the mice inoculated with embryo suspensions of the subsequent egg passage became paralyzed.

Experiment 4

In experiment 4, a larger inoculum was chosen (0.1 ml.) and 9-day chicken embryos were inoculated. The harvest consisted of (1), amniotic fluids and (2), embryos brains and spinal columns. The amniotic fluids (undiluted), and brains and spinal columns were ground to a 20 per cent suspension in saline and were inoculated into mice and eggs on the same day as they were harvested to avoid storage of the egg passage material.

Eight of ten mice inoculated with amniotic fluids became paralyzed with 7 - 17 days. Only one mouse inoculated with the brain and spinal column suspension became paralyzed.

In the subsequent egg passages, all the CNS suspensions were pooled, and all the amniotic fluids were pooled from each passage, and passed separately into eggs and mice. Mice were inoculated with suspensions of subsequent egg passages but none became paralyzed.

Experiment 5

One ml. of a 10 per cent suspension of mouse passage virus was inoculated into 8-day chicken embryos. Heads, torsos and amniotic fluids were harvested, pooled and ground to a 10 per cent suspension. Again egg and mouse inoculations were made on the same day as the embryos were harvested.

Four of ten mice became paralyzed 16-25 days after inoculation of the suspension from the first egg passage. Suspension from the second and the third egg passages did not produce paralysis in mice, however.

Four embryos of the first egg passage died but only one of the suspensions from these eggs produced paralysis in mice.

Experiment 7

In this experiment, only the chicken embryos dying 2 - 8 days after inoculation were harvested. From each egg, serial egg passages were made. Ten-day chicken embryos were inoculated with 0.1 ml. of a 10 per cent suspension of mouse passage virus. Three of eight embryos died but only one proved to contain virus when mice were inoculated. Ten per cent suspensions of a pool of amniotic fluids and embryos were used for the second egg passage and mouse inoculation. All of the chicken embryos of the second egg passage survived.

Results of Egg Inoculation by the Amniotic Route

No specific gross changes were observed in chicken embryos which had died within 2 - 5 days after inoculation, or in embryos which survived this period, even when virus was recovered from the egg passage suspensions. Virus was recovered from suspensions prepared from the chicken embryos which survived the five-day period after inoculation, as well as from a few embryos dying within this period. It was impossible, however, to correlate death of the chicken embryo with presence of the virus.

C. Experiments in which the Intracerebral Route of Inoculation was Used

Four experiments were carried out using the intracerebral route of inoculation and each of these included 3 to 5 serial egg passages. Seven to eleven-day chicken embryos were inoculated with 0.03 ml. of a 10 per cent or 20 per cent suspension of mouse cords and medulla then incubated from 3 - 5 days at 35°C.

Technique for the Intracerebral Method

Eggs are candled and opened over the air sac. The egg shell membrane is removed carefully (as described under the amniotic route method). A 5/8 inch, 27-gauge needle and a tuberculin syringe is used for inoculation. When the head is located, the needle is passed carefully through the chorioallantoic sac and the amniotic sac, then by a sharp thrust, the needle is forced into the cranium just posterior to the eye. With practice, the intracerebral inoculation may be made with only occasional death of the embryo due to trauma.

Material Harvested from the Eggs and Preparation of this Material for the next Egg Passage

Material harvested from experiments 10 - 12 included (1), brains and spinal columns, (2), amniotic fluids and brains and spinal columns, (3), amniotic fluids and heads and torsos. These pools were ground to a 10 per cent or 20 per cent suspension, centrifuged and the supernatant fluids were tested for sterility with blood plates and N.I.H. thioglycolate sterility broth.

Selection of the embryonated egg material for serial egg passage was made in the same way for the intracerebral route passages as for passages in which the amniotic route was used.

Results of the Experiments using the Intracerebral Route

No specific changes were observed in the chicken embryo harvested

after intracerebral inoculation, either in embryos dying within 2 - 5 days after the inoculation or in embryos surviving this period.

None of the mice inoculated with suspensions from any of these egg passages became paralyzed.

D. An Attempt to Investigate the Possibility of the Virus Propagating in Chicken Embryos but Losing Infectivity for Mice

An attempt was made to investigate the possibility that there was propagation of Lansing strain virus which became non-infective for mice after serial egg passage and therefore could not be detected.

For this purpose, it was attempted to immunize mice by repeated intraperitoneal inoculation of various egg passage suspensions from experiments 4 - 8, passages 2 - 5, in which the amniotic route was used and from experiments 10 - 12, passages 2 - 5, in which the intracerebral route was used. The mice were challenged with 0.03 ml. of a suspension of Lansing strain mouse passage virus. The challenge inoculum represented two LD_{50} doses.

There was no protection. This would indicate that there was no virus in the egg material or that the egg suspensions were not sufficiently antigenic to produce, with the amounts inoculated, protection against an intracerebral challenge with infective Lansing strain virus.

E. Experiments in which a Latent Neurotropic Mouse Virus was Accidentally Propagated in the Chicken Embryo

Evidence was found in experiments 9 (amniotic sac inoculation) and 13 (intracerebral inoculation) showing that a latent, neurotropic mouse virus had been picked up from the mouse passage (AM14K1M48) suspension which was used for the first egg passage inoculum. When mice were inoculated with egg passage material, a few developed paralysis, but most of them showed encephalitic symptoms which were not characteristic for Lansing strain poliomyelitis. These mice showed tremors, ruffled fur, weakness of one or more legs and occasionally paralysis of the toes but not of the leg or foot. Death of the mice occurred within 24 hours of the onset of symptoms. A few mice did not show encephalitic symptoms but developed convulsions, with stiffening of the legs, which were followed immediately by death.

In case these symptoms were caused by a bacterial infection, each sterility test was repeated before a suspension was used for egg or mouse inoculation. All suspensions were bacteriologically sterile when harvested except from one egg passage, (experiment 15, passage 5). This suspension was treated with 1000 units penicillin and 8000 units streptomycin per ml. inoculum.

Description of Experiments

Experiment 9: Amniotic Route

Eight-day chicken embryos were inoculated with 0.25 ml. of a 10 percent suspension of mouse passage virus AM14K1M48. Amniotic fluids were harvested for egg and mouse inoculation. Three of eight mice became paralyzed 11 - 13 days after inoculation, and one mouse presented encephalitic rather than paralytic symptoms on the fourth day. Amniotic fluids were harvested from the subsequent egg passages (4 passages in all) for egg and mouse inoculation. All of the mice remained well (see Table II).

Experiment 13: Intracerebral Inoculation

Eight-day chicken embryos were inoculated with 0.03 ml. of a 10 per cent suspension of mouse cords and medullae from passage AM14K1M48. Amniotic fluids and brains were harvested and pooled for the second egg passage. In subsequent egg passages, amniotic fluids and brains were harvested from the living embryos and brains, viscera and amniotic fluid from the dead embryos but remaining in good condition after death. In a few cases, the embryos were too small or disintegration of the tissues had begun and it was necessary to harvest chorioallantoic membranes and allantoic fluids as well.

Results of Mouse Inoculation with Egg Passage Material from Experiment 13

Egg Passage 1: When mice were inoculated with suspensions from this passage, three of eight mice became paralyzed after 3 - 5 days. Three others died within the same period, without symptoms, and one mouse showed a questionable paralysis four days after the inoculation and died within 24 hours. Since the paralysis which did occur was the same as that produced by the Lansing strain, there was no definite indication that another virus was mixed with the Lansing strain from the results of mouse inoculation with the material harvested from the first egg passage.

Egg Passage 2: When mice were inoculated with suspensions from both living and dead chicken embryos, they developed encephalitic rather than paralytic symptoms, within a period of 3 - 5 days. These mice showed tremors, ruffled fur, spasticity and in two cases, paralysis of the toes of one foot but not of the foot or leg. Death of the mice occurred within 24 hours.

Brains, medullae and cords were removed from these mice for a second mouse inoculation. Sterility tests with blood plates and N.I.H. thioglycolate broth showed that each suspension was sterile. These

suspensions were pooled and mice were inoculated. Two to four days after the inoculation, the mice from the second mouse passage showed encephalitic symptoms, tremors and spasticity, but none showed paralysis.

Mice inoculated with egg passage suspensions from passages 3 - 5 also showed encephalitic rather than paralytic symptoms with the exception of one mouse which showed definite paralysis (see Table III - Mouse Inoculation after Egg Passage 5, experiment 13).

Attempts to Differentiate between the Lansing Virus and the Virus which Produced Encephalitic Symptoms in Mice

In an attempt to differentiate between the Lansing virus and the virus which produced encephalitic symptoms in mice, neutralization tests were carried out with CNS suspensions from mice showing encephalitic symptoms and from a mouse which developed paralysis. These mice had been inoculated with egg passage material from experiment 13, passage 4, (see Table II). The serum chosen came from a pool of normal human serum gamma globulin which had been used previously in immunological work to neutralize the Lansing strain. Normal horse serum was used for the controls.

There was no difference in the incidence of paralysis or of encephalitic symptoms in the mice inoculated with either one of the CNS suspensions.

The human serum gamma globulin neutralized the virus in both suspensions. The LD_{50} of the virus with normal human serum gamma globulin was .003 per cent for CNS suspension from mice showing encephalitic symptoms. The LD_{50} for the virus with normal horse serum was over .0001 per cent (end point was not reached).

If neutralization of the virus had not occurred it would have been an indication that the virus was not related to the Lansing strain. The fact that neutralization did occur was not considered to be a positive finding, however because normal human serum may contain antibodies or a

non-specific inhibitor for viruses other than the Lansing strain. Neutralization tests using a specific anti-Lansing serum were not carried out because this antiserum was not available at the time.

A further attempt was made to differentiate between the viruses by immunizing mice with the Lansing strain and challenging with the virus which produced encephalitic symptoms in mice.[1] Ten mice were inoculated intraperitoneally with 0.2 ml. of a 10^{-1} dilution of a Lansing strain. Two weeks after the last inoculation, they were challenged intracerebrally with 0.03 ml. of a 10^{-4} dilution of the virus which produced encephalitic symptoms. Ten control mice were inoculated intracerebrally with 0.03 ml. of a 10^{-4} dilution of the same virus suspension.

In both groups, 8 of the 10 mice inoculated developed encephalitic symptoms 3 - 6 days after the inoculation. Thus, in this experiment, immunization with the Lansing strain virus gave no protection against the virus which produced the encephalitic symptoms in mice.

After the results of mouse inoculation in experiments 9 - 13 were observed, mice were inoculated with AM14K1M48 passage of Lansing strain again. The mice were checked very carefully and four of the mice inoculated showed encephalitic symptoms, tremors, ruffled fur, weakness of the legs, etc. Four of the mice showed symptoms typical for Lansing strain.

Embryonated eggs were inoculated by the amniotic sac and intracerebral routes with AM14K1M48 passage of the Lansing strain and mice were inoculated with suspensions prepared from the first egg passage. Again some of the mice showed paralysis typical for Lansing strain and some showed encephalitic symptoms.

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1. This procedure was based on data published by Kramer and Geer (1945)[40]

Mice were inoculated with spinal cord suspensions from passages AM14K1M46 and AM14K1M47 and all mice showed paralysis typical for Lansing strain of poliomyelitis virus. It seemed evident, therefore, that another virus was present in the mouse passage (AM14K1M48) suspension.

DISCUSSION

The data presented show that when the amniotic sac method of inoculation was used, the Lansing strain of the poliomyelitis virus was recovered only after the first egg passage. Since the LD_{50} titer of the virus was much higher than the virus dilution obtained when 0.1 ml. of a 10 per cent suspension of mouse passage virus was inoculated into the amniotic sac, it seemed probable that the virus was passively transferred in the first egg passage rather than that proliferation of the virus occurred.

In two experiments in which the amniotic and intracerebral routes were used, some of the mice inoculated with suspensions from the first egg passage showed encephalitic symptoms and some showed paralysis. Most of the mice inoculated with suspensions from subsequent egg passages in the experiment in which the intracerebral route was used developed encephalitic symptoms. Since the same mouse passage suspension was used for the first egg passage in both experiments, this suspension was checked by mouse inoculation. Half of the mice showed paralysis and half showed encephalitic symptoms. Mice were inoculated with a suspension from the preceeding mouse passage and all showed paralysis typical for the Lansing strain. It seemed evident, therefore, that the virus which produced encephalitic symptoms in mice was picked up accidentally when mouse cords were collected for the AM14K1M48 Lansing virus passage.

An attempt was made to differentiate between the Lansing virus and the virus which produced encephalitic symptoms by using neutralization tests with normal human serum gamma globulin. There was neutralization of the virus which produced encephalitic symptoms, but it was not considered as a positive finding because normal human serum may contain antibodies or a non-specific inhibitor for viruses other than the Lansing strain.

When mice were immunized with Lansing strain and challenged with the encephalitic producing virus, the incidence of encephalitis was the same in this group as in the control group which were not immunized with the Lansing strain.

In view of the different symptoms produced in the mice, and the fact that immunization with the Lansing strain did not protect mice when challenged with this virus, it seemed evident that it had been picked up accidentally from the mouse cord suspension used for the first egg passage.

SUMMARY

An attempt to propagate the Lansing strain of poliomyelitis virus in developing chicken eggs has been made in this study. Both the amniotic and intracerebral routes were used. Three to five serial egg passages were carried out in each experiment. Whenever possible, storage of the mouse cords and egg passage material was avoided, and suspensions were prepared and inoculated the same day on which they were harvested.

Lansing virus was recovered only from the first egg passage in the experiments in which the amniotic route was used. It seemed probable, therefore, that this was due to passive transfer rather than proliferation of the virus.

Lansing strain of poliomyelitis virus was not recovered from any of the experiments in which the intracerebral route was used.

In one experiment in which the intracerebral route was used, a latent, neurotropic mouse virus was accidentally picked up and propagated through five serial egg passages.

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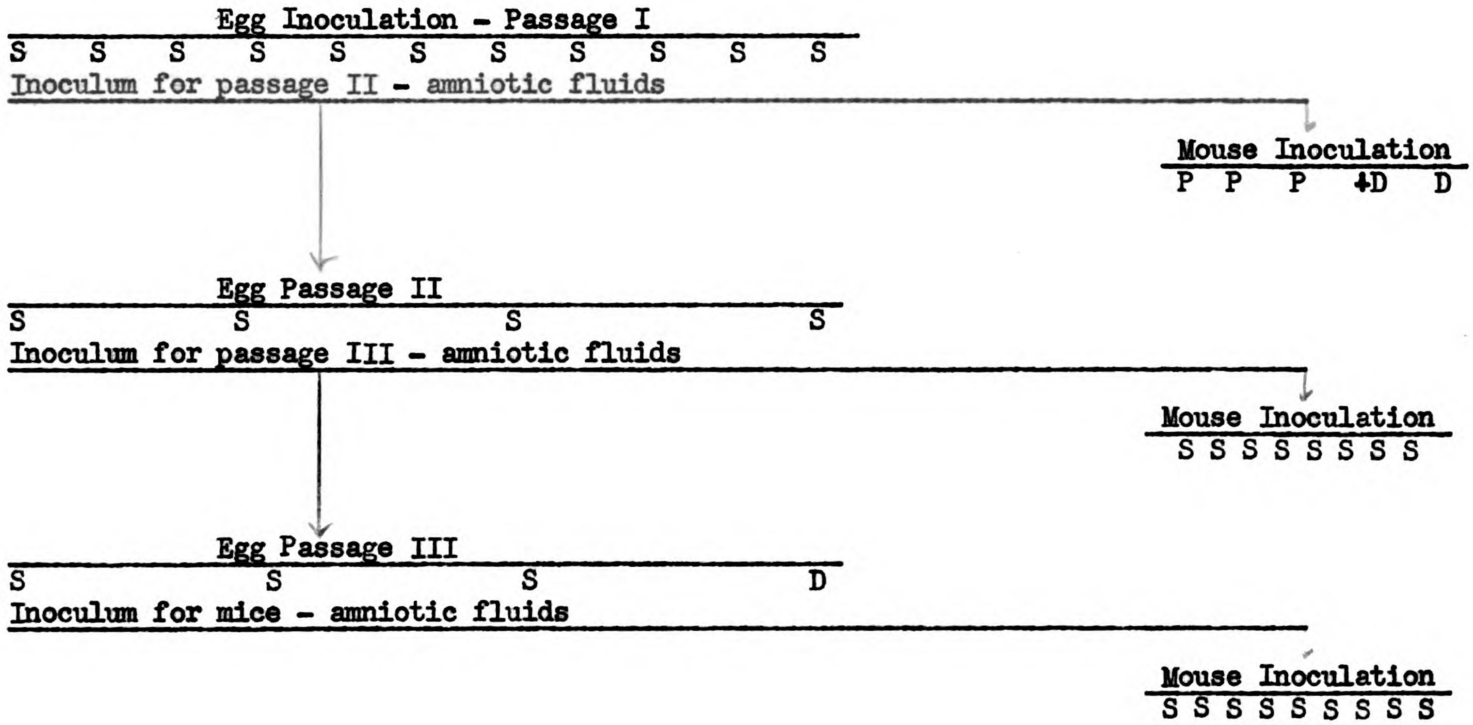
TABLE II

EXPERIMENT #9 WITH THE LANSING STRAIN PASSAGE $AM^{14}K^{1}M^{48}$
METHOD OF INOCULATION: AMNIOTIC ROUTE

Egg Passage I - 8-day chick embryos inoculated with 0.25 ml. of a 10% suspension of mouse cords and medullae



Note: S - surviving mouse or chick embryo
D - dead
P - mouse showing flaccid paralysis
+D - mouse showing encephalitic symptoms such as tremors, weakness of legs and sometimes convulsions, followed by death within 24 hours.

TABLE III

EXPERIMENT #13 WITH THE LANSING STRAIN PASSAGE $AM^{14}K^{1}M^{48}$
METHOD OF INOCULATION - THE INTRACEREBRAL ROUTE

Egg Passage I -8-day chick embryos were inoculated intracerebrally with 0.03 ml. of a 10% suspension of mouse cords and medulla from passage $AM^{14}K^{1}M^{48}$

Egg Inoculation

S S S S S S S

Inoculum for egg passage II
(1) 10% susp. brains, spinal columns, (living embryos)
(2) 10% susp. brains, spinal columns, (dead embryos)

Mouse Inoculation

P P P +D D D D

Egg Passage II

S S S D D D

Inoculum for egg passage III
(1) 10% susp. brains, spinal columns, (living embryos)
(2) 10% susp. brains, abdominal viscera, extra embryonic fluids (dead embryos

Mouse Inoculation(I)

+D +D +D +D +D +D +D

Mouse Inoculation (2)

+D +D +D +D +D +D +D

Egg Passage III

(1) S S S S S D (2) S D D D

Inoculum for egg passage IV
(1) 10% susp. brains, spinal columns, (living embryos)
(2) 10% susp. brains, abdominal viscera, allontoic fluids (dead embryos)

Mouse Inoculation (1)

+D +D D D D

Mouse Inoculation (2)

S S S S S

Egg Passage IV

(1) S S S S D D D D (2) S D D D

Inoculum for egg passage V
(1) 10% susp. brains, spinal columns, (living embryos)
(2) 10% susp. viscera brains, amniotic fluids (dead embryos)

Mouse Inoculation (1)

P +D +D +D +D

Mouse Inoculation (2)

S S S S

Egg Passage V

(1) S S S S S S S D D D D D (2) S S S S S S S S S D D D

Inoculum for mouse inoculation
(1) 10% susp. brains, amniotic fluids (living embryos) (treated with antibiotics because of bacterial contamination)
(2) 10% susp. abdominal viscera, brains, amniotic flds. (dead embryos) (treated with antibiotics because of contamination)

Mouse Inoculation (1)

S S S S S

Mouse Inoculation (2)

+D +D +D S D

Note: S - surviving mouse or chick embryo
D - dead
P - mouse showing flaccid paralysis
+D - mouse showing encephalitic sympotoms such as tremors, weakness of legs and sometimes convulsions, followed by death within 24 hours.

TABLE I

EXPERIMENTS CONDUCTED IN AN ATTEMPT TO PROPAGATE THE MOUSE ADAPTED LANSING STRAIN OF POLIOMYELITIS VIRUS IN EMBRYONATED CHICKEN EGGS

	Exp. No.	Embryo Inoculation: Mouse Passage	Embryo Inoculation: Age of Embryo	Embryo Inoculation: Incubation	Embryonic Tissues Harvested: Tissues Harvested	Embryonic Tissues Harvested: Disposition of Egg Harvest	Inoculum for the next egg passage	Results of Mouse Inoculation after Passages in Eggs: Egg Passage I	Egg Passage II	Egg Passage III	Egg Passage IV	Egg Passage V
Amniotic Sac Inoculation Inoculum for First Egg Passage - 0.03 ml. of a 10% susp. of infected mouse cords and medullae	Exp. #1	AM14K1M41	7-9 days	3-5 days at 37°C	Amniotic fluids	(1) Amniotic fluids passed undiluted, (2) tissues ground to a 20% susp. + in saline	0.05 ml.	(1) 0/10+ (2) 0/10	0/10	0/10	0/10	not done
	Exp. #2	AM14K1M41	7-9 days	3-5 days at 37°C	Brains, spinal columns, abdominal viscera (stored at -30°C)	Ground to a 20% suspension in saline	0.05 ml.	0/10	0/10	0/10	not done	not done
	Exp. #3	AM14K1M41	7 days	3-5 days at 37°C	Heads and torsos (stored at -30°C)	(1) Ground to a 20% suspension in saline (2) 20% susp. concentrated 7 times	0.05 ml.	(1) 1/10 (2) 1/10	0/10	0/10	0/10	not done
Amniotic Sac Inoculation Inoculum for First Egg Passage - 0.1 ml. of a 10% susp. of infected mouse cords and medullae	Exp. #4	AM14K1M46	7-9 days	4-5 days at 35°C	Amniotic fluids, brains and spinal columns, (passed on the same day)	(1) Amniotic fluids passed undiluted, (2) tissues ground to a 20% susp. in saline	0.1 ml.	(1) 8/10 (2) 0/10	0/10 0/10	0/10 0/10	0/10 0/10	0/10 0/10
	Exp. #5	AM14K1M46	8-9 days	3-4 days at 35°C	Amniotic fluids pooled with heads & torsos (passed on the same day)	Ground to a 20% susp. in saline	0.1 ml.	4/10 1/20++	0/10	0/10	0/10	0/10
	Exp. #6	AM14K1M46	1st Pass. 11 days Other Pass. 7-9 days	3-4 days at 35°C	Amniotic fluids pooled with heads & torsos (passed on the same day)	Ground to a 20% susp. in saline	0.1 ml.	0/10	0/10	0/10	not done	not done
	Exp. #7	AM14K1M46	7-10 days	3-4 days at 35°C	Amniotic fluids pooled with heads & torsos (passed on the same day)	Ground to a 20% susp. in saline	0.1 ml.	0/10	0/10	0/10	0/10	0/10
	Exp. #8	AM14K1M46	10 days	2-8 days at 35°C	Only 3 dead embryos harvested Amniotic fluids pooled with heads & torsos (passed on the same day)	Ground to a 20% susp. in saline	0.1 ml.	(1) 0/10 (2) 0/10 (3) 1/10	0/10 0/10 0/10	not done	not done	not done
Intracerebral Inoculation Inoculum for First Egg Passage - 0.03 ml. of a 10% susp. of infected mouse cords and medullae	Exp. #10	AM14K1M46	1st Pass. 11 days Other Pass. 7-9 days	3-4 days at 35°C	Brains, spinal columns, (passed on the same day)	Ground to a 20% susp. in saline	0.03 ml.	0/10	0/10	0/10	0/10	0/10
	Exp. #11	AM14K1M46	7-10 days	3-4 days at 35°C	Amniotic fluids, heads and torsos pooled (passed on the same day)	Ground to a 20% susp. in saline	0.03 ml.	0/10	0/10	0/10	0/10	0/10
	Exp. #12	AM14K1M46	7-10 days	3-4 days at 35°C	Amniotic fluids pooled with heads & torsos or spinal column (passed on the same day)	Ground to a 20% susp. in saline	0.03 ml.	0/10	0/10	not done	not done	not done

\+ 0/10; numerator - number of mice paralyzed
denominator - total number of mice inoculated

++ 1/20; suspensions from 4 dead embryos in the first passage were each inoculated into 5 mice - only one of the suspensions produced paralysis

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