STUDIES ON THE PURIFICATION OF RABIES VACCINE DERIVED FROM RABBIT BRAIN

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by

Robert J. Gauthier

AN ABSTRACT

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

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Department of Microbiology and Public Health

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It has long been known that antirabic vaccines occasionally produce allergic encephalitis or other severe central nervous system reactions in vaccinated individuals. Although the cause of these reactions is not clear, most workers believe it to be an allergic reaction to the brain tissue contained in the vaccine. It is generally believed that post-vaccinal reactions could be greatly reduced if the vaccines were freed of most of the non-specific brain tissue. The vaccines must, however, be highly antigenic.

This investigation was undertaken to develop an acceptable method for the purification of rabies vaccine derived from rabbit brain. Our procedures employed zinc precipitation of the antigen followed by selective dissociation of the zinc complex.

These procedures, which incorporated pH changes, failed to purify the rabies vaccine without appreciably decreasing its antigenicity. Although attempts to purify the vaccine were unsuccessful, significant data were obtained concerning the nature of the rabies antigen.

Under the conditions employed, it was found that the rabies antigen was largely insoluble or poorly dispersed in aqueous and saline media. This was thought to be due either to the intimate association of the antigen with the brain substances or to its chemical composition. In any case, this insolubility was the apparent reason for the inability of the zinc to precipitate the bulk of the antigen from the vaccine.

Sonic oscillation, under the conditions employed, greatly increased the antigenicity of the rabies vaccine. However, no comparable increase

was found in its antigen solubility. The liberation of some insoluble antigen, which was zinc precipitable, followed sonic disintegration of the tissue cells.

Repeated freezing and thawing of the rabies vaccine resulted in the complete destruction of the antigenic component. Similarly, the extraction of the vaccine with ether or a mixture of ether and ethanol at low temperatures resulted in a great loss of antigenicity. There appears to be some similarity between the rabies antigen and lipid-protein combinations.

It was concluded that before any acceptable method of rabies vaccine purification could be developed, it will be necessary first to more adequately determine the chemical properties of the inactivated rabies virus. The intimate association of the antigen with brain substances or protective chemical complexes has defied most physical and chemical methods of separation without denaturation of the antigen.

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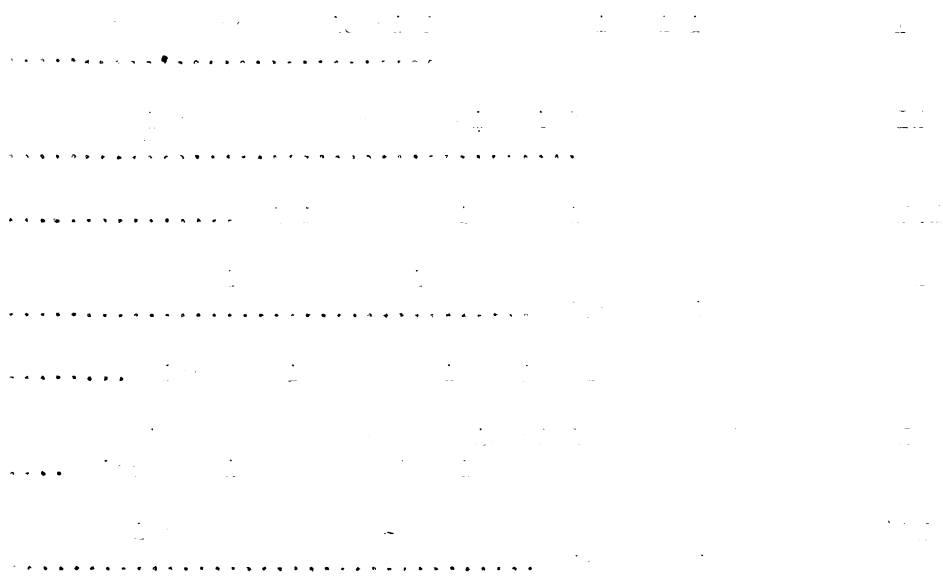


I. INTRODUCTION

One of the main problems of rabies research is to produce a vaccine which will be highly antigenic and free from the agents which cause postvaccinal reactions.

It has long been known that antirabic vaccines occasionally produce paralytic accidents. The cause of these reactions is not clear; however, most workers believe it to be an allergic reaction to the brain tissue contained in the vaccine. Results of the studies of Stuart et al. (1928), Rivers et al. (1933), Lewis (1933) and Kabat et al. (1947) show that brain tissue functions as an organ-specific instead of a species-specific antigen. Paralysis caused by rabies vaccination must, therefore, be considered as a specific sensitization to brain material. It is generally believed that the maximum reduction in postvaccinal reactions could be obtained if the non-specific brain tissue could be removed from the vaccine. The vaccine must, however, be highly antigenic.

The zinc precipitation method for the fractionation of plasma described by Cohn (1953) was modified by Martin (1954), Chapman (1954), and Smolens (1955) for the purification and concentration of viruses.

Cox et al. (1947) reported rabies virus purification by means of alcohol precipitation. Harris (1948) employed ether extraction and Bell et al. (1949) ether-benzene extraction for the purification of rabies virus.

The purpose of this study is to investigate possible methods of rabies vaccine purification employing some of the virus purification procedures.

II. HISTORICAL REVIEW

Rabies has been known in Europe and Asia since ancient times. Rabies was described in dogs and domestic animals by Aristotle. He observed that other dogs bitten by rabid dogs likewise became made. Galen (200 A.D.) gave one of the earliest medical descriptions of the disease as follows: "Hydrophobia is a disease that follows the bite of a mad dog and is accompanied by an aversion to drink liquids, convulsions and hiccoughs. Sometimes maniacal attacks supervene" (Castiglioni, 1941).

According to Johnson (1948) rabies was known in western Europe as early as 1271, at which time it was prevalent among wolves in France. The disease appeared in Italy in 1708 in epizootic proportions in dogs and by 1728 spread to most of the major cities of Hungary, Germany and France. Mullett (1945) stated that rabies was known in England in 1613, but not in epizootic proportions until 1734.

In the United States rabies was known as early as 1753 in Virginia, 1762 in North Carolina and by 1785 spread through New England (Johnson, 1948).

Rabies was shown to be infectious by Zinke (1804) by the inoculation of saliva of rabid dogs into normal dogs. The work of Zinke, cited by Webster (1942) was not available for study. Orfila (1817) specifically incriminated the saliva as the source of rabies infection (Mettler, 1947). Galtier (1879) introduced the use of domesticated rabbits for the study of rabies.

Pasteur (1885) successfully developed a rabies prophylaxis consisting of the subcutaneous injection of a fixed attenuated virus preparation obtained from an emulsion of the spinal cords of rabbits dead of rabies. This vaccine was adopted as a routine procedure in medical centers throughout the world.

Many modifications of the Pasteur method have been developed since that time. Among these have been the dilution method of Högyes (1897) and the glycerol containing vaccine of Calmette (1891).

Fermi (1908) introduced the use of phenol in the treatment of tissue suspensions of fixed virus for the production of a vaccine. Semple (1911) produced a phenol killed rabies vaccine that was completely non-infectious and yet effective as an immunizing agent.

It was soon determined, however, that rabies vaccine could occasionally produce an allergic encephalitis or other severe central nervous system reactions in vaccinated individuals.

The incidence of paralytic accidents as a result of antirabic vaccination was shown by McKendrick (1940) to be one in 8,887 for phenol-killed virus vaccines, one in 3,398 for the attenuated virus vaccines and one in 3,194 for diluted virus vaccines.

In the tenth analytical report on antirabies treatment the occurrence of postvaccinal paralysis was shown by Greenwood (1945) to be one in 8,517 for phenol-killed virus vaccines, one in 3,375 for the attenuated virus vaccines and one in 3,435 for the diluted virus vaccines.

Redewill and Underwood (1947) report the incidence of severe postvaccinal complication following rabies vaccination at one per 1,194 persons treated in Los Angeles County during a five year period (1940-1945 inclusive).

In Los Angeles County and City, Pait and Pearson (1949) reported nine cases of postvaccinal encephalitis among 5,500 treated persons, an incidence of one in 600. The authors indicated that in this area the possibility of acquiring postvaccinal reactions as a result of antirabies treatment is approximately twice as great as that of acquiring rabies from known dog bites.

Cook et al. (1955) reported that postvaccinal reactions occurred at the rate of one in 527 treated persons in a group of 8,430 treated with phenolized rabies vaccine in Texas from 1949 through 1953. Appelbaum (1953) reported the incidence of encephalomyelitis as a result of rabies vaccination at one accident to 2,025 persons treated in New York City Department of Health Clinics. Here the risk of rabies from a bite was shown by the author to be greater than that of encephalitis from vaccination.

It is generally believed, however, that many minor cases of paralytic accidents fail to be reported and that the true incidence is higher than any published data would indicate (Remlinger, 1927).

Published statistical data on the occurrence of postvaccinal reactions following rabies vaccination in Michigan was not available.

Although rabies vaccines containing nerve tissue have been used extensively, they cannot be considered entirely safe for use in either man or animal since myelitis or encephalitis occasionally occurs (Burkhart et al. 1950). The possibility of such reactions from the injection of nerve tissue must be balanced against any advantage attained by the use of such vaccines.

The vaccine which has been most widely employed within recent years has been the Semple type vaccine, which is a killed vaccine prepared from brain tissue, the inactivating agent being phenol. This is, of course, one of the crudest types of antigenic material.

The cause of paralytic accidents resulting from antirabic treatment have, in the past, been ascribed to infection with the fixed virus contained in the vaccine by Fielder (1916), Busson (1926) and Van Stockum (1935). Bassoe and Grinker (1930), however, believed the reactions were due to a separate virus entity. Marsden and Hurst (1932) also stated that the postvaccinal reaction is entitled to recognition as an independent clinical and pathological entity. It was observed, however, that these reactions continued to occur even when completely killed vaccines were employed.

Some of the earlier workers, on the other hand, were of the opinion that the reactions were of an allergic nature. Stuart and Krikorian (1928) believed that the reactions resulted from the introduction of nerve substance during the process of immunization. Stimson (1910) suggested that it was an allergic reaction to foreign nerve protein, while Cornwall (1918) stated that the reactions were anaphylactic in nature, the antigen being either normal brain matter or the products of metabolism of the rabies organism.

It has more recently been indicated that the encephalitic reactions are allergic in nature. The neurologic lesions are believed to arise from an allergic reaction of the patient to the nervous tissue in the vaccine.

In determining the incidence of allergy among those receiving rabies prophylaxis, Horack (1939) was able to demonstrate allergy in 87.5 per cent of the paralytic cases and 33 per cent in cases showing no neurological symptoms.

Symptoms and pathological changes in the central nervous system, similar to those produced in postvaccinal reactions, were shown by Freund et al. (1947) to result from serial injections of normal brain tissue with adjuvants in guinea pigs. Similar results were obtained by Jervis and Koprowski (1948) and Koprowski and Jervis (1948).

Some workers have been able to produce paralysis in animals by means of repeated injections of either homologous or heterologous brain material. Schwentker and Rivers (1934) demonstrated paralysis in rabbits by means of repeated injections of normal rabbit brain tissue. Kabat et al. (1947) and Morgan (1947) were able to show encephalomyelitis in monkeys as a result of injections with normal monkey brain. Rivers, Sprunt and Berry (1933) and Rivers and Schwentker (1935) reported encephalomyelitis in monkeys as a result of injections of normal rabbit brain and extracts of normal rabbit brain.

Olitsky and Tal (1952) and Tal and Olitsky (1952), however, have shown that the proteolipides, isolated from brain by Folch and Lees (1951a, 1951b) are capable of bringing about acute disseminated encephalomyelitis in mice indistinguishable from the condition induced by the inoculation of whole brain tissue. Goldstein et al. (1953) demonstrated the same reaction with guinea pigs.

There are indications that the postvaccinal reactions are due to antibody to the injected brain material reacting with the tissues of the nervous system of the susceptible animal, thereby producing the encephalitis or other nervous system reaction.

Schwentker and Rivers (1934) determined that brain tissue under proper conditions functions as a complete antigen and is capable of producing complement-fixating antibodies in rabbits which are organ-specific

rather than species-specific. Lewis (1933) demonstrated the presence of anti-brain antibodies in experimental animals injected with brain substance. These antibodies to brain tissue were also shown by Kirk and Ecker (1949) to be produced in humans receiving antirabies vaccine. Koprowski and LeBell (1950) demonstrated the development of complement-fixating antibodies to brain tissue in the serums of 50 per cent of 34 persons receiving Semple vaccine.

These observations strengthen the hypothesis that postvaccinal reactions are in some manner associated with the development of specific antibodies for brain.

The cause of the postvaccinal reactions is not clear. However, it is believed that either the removal of the encephalogenic factor from the vaccine or the use of a vaccine which does not contain brain tissue would probably eliminate the danger of such reactions (Jervis, 1954).

Rabies virus obtained from suspensions of infected tissue may be purified to some degree by the common methods of selective precipitation of serum proteins. Cox et al. (1947) reported purification and concentration of rabies virus by means of alcohol precipitation. Tagaya et al. (1953), however, does not recommend alcohol precipitation because repeated treatment results in a great loss of virus activity, but favors repeated acid precipitation. Behrens et al. (1939) reported purification of rabies virus by precipitating the tissue protein at its isoelectric point.

In addition, Warren et al. (1949) succeeded in precipitating rabies virus with protamine sulphate and Muller (1950) and Sawai et al. (1954) purified rabies virus with the application of ion exchange resins.

Apparently none of the foregoing methods of rabies virus purification has been adaptable to quantity production of purified rabies vaccines.

Bell, Wright and Habel (1949), however, described a method for the removal of the encephalogenic factor from brain tissue suspensions by the fractionation of benzene-ether treated infected brain tissue with calcium acetate. Paterson et al. (1953) indicated that protamine sulfate appeared to sediment most, if not all, of the encephalogenic activity of both rabbit and mouse brain material. Harris (1948) employed ether at low temperatures to extract the fats and lipids from infected brain material. D'Silva et al. (1951) and Hottle and Peers (1954), however, advocate the use of centrifugation of brain tissue suspensions in distilled water to remove a large part of the encephalogenic factor from rabies vaccine.

Cohn and his workers (1953) successfully purified and fractionated the various important proteins of human plasma employing the interaction of the proteins with heavy metals. Chapman and Surgenor (1954) utilized this method for the precipitation, concentration and purification of viruses. The method proved successful for the extraction of several kinds of viruses from several kinds of tissues and tissue fluids. With the zinc precipitation method, they were able successfully to precipitate and extract the Rous sarcoma virus from infected allantoic and amniotic fluids and from aqueous and saline extracts of tumors, embryonic liver, chorioallantoic and amniotic membranes. This method was modified by Martin and Chapman (1954) to purify and concentrate the PR8 influenza virus from infected allantoic fluids. In addition, modifications of

this method have been applied to the concentration and purification of Polio viruses from kidney tissue by Smolens et al. (1955).

It is generally assumed that viruses are protein in nature. Therefore, it should be possible to separate them from tissues by the same method which enabled the fractionation of plasma. Some viruses can be concentrated from infected tissues as metal complexes and following the dissociation of the metal-virus complex, the virus is obtained without measurable loss of yield or potency.

The use of brain tissue in rabies vaccine production is both practical and economical. The method of purification of rabies vaccine should, therefore, be amenable to the use of brain tissue as the starting material even though it contains excessive amounts of extraneous material and a high concentration of nonviral materials which probably have properties similar to those of the virus.

It is not within the scope of this study to purify the living rabies virus per se, but rather to reduce the concentration of non-specific materials contained in the vaccine without appreciably decreasing its antigenicity.

III. MATERIALS AND METHODS

A. Rabies Virus

The fixed virus strain employed in this study was received from the Department of Health in Detroit, Michigan in 1932. The Detroit Department of Health obtained the virus from Dr. F. G. Novy of the Pasteur Institute at the University of Michigan in Ann Arbor, Michigan, who in turn received the virus from Dr. Louis Pasteur. The virus employed represents 196 passages at the Michigan Department of Health.

The virus was prepared as an emulsion containing 20 per cent rabbit brain tissue in a diluent of two per cent horse serum in distilled water. The infected rabbit brain was ground in a chilled Waring Blendor for five minutes and dispensed in 3-ml amounts in ampoules. The ampoules were flame-sealed and the contents were shell frozen in a dry ice-alcohol bath. The ampoules were stored at -50°C. until needed.

When needed an ampoule was removed from storage, the contents thawed and 2.5 ml of the emulsion was diluted with 2.5 ml of two per cent horse serum in distilled water to give a ten per cent tissue concentration. This suspension was centrifuged at 3500 r.p.m. for 15 minutes and the supernate was drawn off and diluted to 1×10^{-3} for rabbit passage.

B. Preparation of Rabies Vaccine

Normal adult rabbits were injected intracerebrally through the opening about one-half inch posterior to the outer canthus of the eye with 0.2 ml of a 1×10^{-3} dilution of the fixed virus by means of a 20 gauge needle

with a short bevel. After the rabbits showed symptoms of rabies and had been prostrate for 24 hours, which was usually on the fourth and fifth day after inoculation, they were sacrificed by injecting air into the ear vein.

The body of the rabbit was then sheathed in a cloth saturated in five per cent phenol solution and the hair on the head was also wet down with five per cent phenol solution.

An incision was made down the mid-line of the head and the skin folded back. Care was exercised not to cut the ear canals, inasmuch as large numbers of bacteria are present in this area. The skull was opened by means of bone forceps, the brain removed and placed in a sterile tared jar. Each brain was tested for sterility by removing a small piece of brain tissue from each of two different portions of the brain and placing them into tubes of National Institutes of Health sterility test medium. After sterility tubes had been seeded, the brain was weighed and placed in storage at -50°C.

The infected brains, kept in glass jars, were thawed in a bath of five per cent phenol solution and then transferred to the sterile hopper of a colloid mill, the rotor of the colloid mill having been previously sterilized with ten per cent phenol and thoroughly rinsed with sterile distilled water.

A portable circulating refrigeration bath was connected to the mill and cold (-5°C.) ethylene glycol circulated through the hopper jacket.

Sufficient saline was also placed in the mill hopper to give a 50 per cent tissue emulsion. The brains were emulsified by grinding for two minutes. The suspension was then sampled to determine its virus content. After sampling, a calculated volume of phenolized saline was added to the 50 per cent emulsion in a hopper and the grinding continued for another two

minutes. This 40 per cent emulsion, containing 0.8 per cent phenol, was drained from the mill hopper into gallon bottles. Merthiolate was added to a concentration of 1:10,000 and the 40 per cent brain emulsion was allowed to attenuate at room temperature (20-25°C.) for 21 days. The emulsion was agitated gently several times daily.

After 21 days the vaccine was diluted to 6.66 per cent tissue with 0.85 per cent saline and screened through 100 and 150-mesh monel metal screens. Additional merthiolate was added to give a final concentration of 1:10,000.

The vaccine was sampled and six mice were injected intracerebrally with 0.03 ml to determine if complete attenuation had occurred. The mice were observed for symptoms of fixed virus rabies for a period of 14 days. When the virus was shown to be non-viable by mouse test, the vaccine was sampled for antigenicity and safety test in rabbits. Two rabbits were injected intracerebrally with 0.25 ml each in the same manner as used for injection of rabbits for production purpose. The rabbits were observed 14 days for symptoms of fixed virus paralysis.

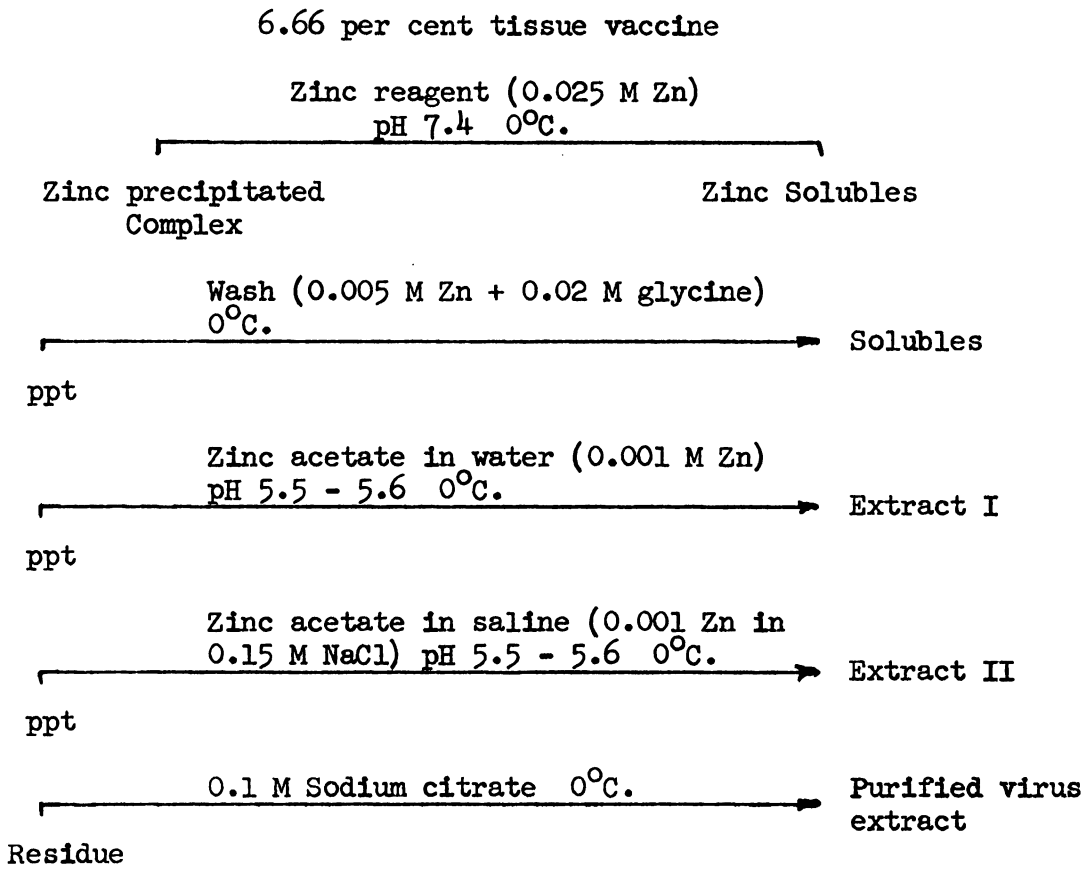
C. Methods of Purification

The purification methods employed in these studies were based upon the use of a 6.66 per cent rabbit brain tissue vaccine as the starting material. This vaccine is representative of that produced by the Michigan Department of Health.

1. Zinc precipitation methods.

   a. Method of Chapman and Surgenor. Lot I was purified according to the tentative method of Chapman and Surgenor (1953) for the extraction and purification of viruses.

A schematic diagram of the procedure is as follows:



All glassware, reagents and vaccine suspensions were kept at 0° to 4°C. throughout the precipitation and extraction procedures. In addition, the entire procedure was carried out in a refrigerated bath and centrifuge. The temperature never exceeded 5°C.

The vaccine suspension was gently stirred and the zinc reagent added dropwise in the ratio of 0.25 ml zinc reagent per 5 ml of vaccine suspension. After the complete addition of the zinc reagent and adjustment of the pH to 7.4 with 1 N NaOH, the mixture was allowed to equilibriate for an hour at 0°C. with occasional stirring. The

mixture was centrifuged at 3000 r.p.m. for 30 minutes and the supernate removed (zinc solubles).

The precipitate was resuspended in the wash reagent at 0°C. and centrifuged at 1500 r.p.m. for 30 minutes and the supernate removed (solubles).

The precipitate was resuspended in the zinc acetate in water solution. The pH was carefully adjusted to 5.5 - 5.6 by bubbling CO_2 through the mixture while the suspension remained at 0°C.* The mixture was centrifuged at 1500 r.p.m. and the supernate removed (Extract I). The precipitate was resuspended in the zinc acetate in saline solution, while the suspension was held at 0°C. The pH was again carefully adjusted to 5.5 - 5.6 by bubbling CO_2 through the mixture. The mixture was centrifuged at 1500 r.p.m. for 30 minutes and the supernate removed (Extract II). The precipitate was dissolved in a 0.1 M sodium citrate solution to extract the antigen from any remaining insoluble materials. The mixture was centrifuged at 3000 r.p.m. and the supernate recovered.

The supernate represented the purified vaccine extract and was designated I-A.

b. Modifications of zinc method. Lots II, III and IV represent certain modifications of the procedure for precipitating and extracting the rabies antigen (virus) as advocated by Chapman and Surgenor.

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* A small CO_2 generator was made by adding water to dry ice and washing the released gas in water.

The precipitation procedure for Lot II was altered. The pH of the starting material was not adjusted to 7.4 with NaOH. The pH of the starting material itself was 6.8.

In processing Lot III, the following modifications were made in the extraction procedure:

Lot III-A. The zinc acetate in water extraction was carried out at pH 5.8.

The zinc acetate in saline extraction was carried out at pH 6.1.

Lot III-B. The zinc acetate in saline extraction was carried out at pH 5.8.

Lot III-C. The zinc acetate in water extraction was carried out at pH 5.8.

Instead of zinc acetate in saline, the precipitate was extracted with buffered saline at pH 5.9.

The precipitation procedure was also altered for Lot IV. The pH of the starting material was adjusted to 7.8 and the diluent for the material was distilled water.

c. Method of Martin and Chapman. A 100 ml sample of 6.66 per cent vaccine was treated in accordance with the procedure of Martin and Chapman (1954) for the purification of influenza virus.

The sample was zinc precipitated in the same manner as described for the method of Chapman and Surgenor. The precipitate, however, was repeatedly extracted with 1 M glycine at pH 7.3. The extraction procedure was carried out first with 100 ml of glycine and then with three successive 40-ml volumes of glycine. The material was centrifuged each time at 3000 r.p.m. for one hour at 3°C. The extracts were designated as Lots V-A, V-B, V-C and V-D, respectively. The residue of the final extraction was made up to the original volume

of the starting material with saline. This residue vaccine was labeled Lot V-E.

d. Vaccine antigen solubility study. A sample of the starting 6.66 per cent tissue vaccine made in saline was centrifuged at 3°C. for 30 minutes at 3500 r.p.m. The supernatant fluid was drawn off and designated Lot A-1.

A sample of the starting vaccine made in distilled water was also centrifuged at 3°C. for 30 minutes at 3500 r.p.m. and the supernatant fluid was drawn off and designated as Lot B-1.

After centrifugation the residues of the above vaccine samples were made up to their original volumes with their respective diluents. These products were designated as Lots A and B, respectively.

2. Physical methods of cellular disintegration.

a. Sonic oscillation. Sonic vibration was applied to 50 ml samples of the vaccines. In order to determine the optimal time exposure to sonic vibration the samples were sonerated at various time intervals. The material was sonerated in a 10-k.c. Raytheon oscillator. The sonic oscillator cup containing the vaccine was continuously cooled by passing cold (-10°C.) ethylene glycol through the oscillator cup jacket.

The time of exposure of the vaccine to sonic vibration was 2, 4, 6, 8, 10 and 14 minutes. These samples were designated as Lots VI-2, VI-4, VI-6, VI-8, VI-10 and VI-14, respectively.

b. Freeze-thaw technique. A 100-ml sample of the vaccine was disintegrated by alternately freezing and thawing. The brain tissue suspension was frozen rapidly by means of an alcohol-dry ice bath,

followed by rapid thawing in a 37°C. water bath. This procedure was repeated 20 times. This material was centrifuged in a refrigerated centrifuge at approximately 3000 r.p.m. for 30 minutes. The supernatant fluid was drawn off and the residue was made up to its original volume with physiological saline. The products were then designated as Lots VII-A and VII-B, respectively.

Slow alternate freezing and thawing of a 100-ml sample of the vaccine was carried out 20 times. The suspension was frozen in a mechanical freezer at -50°C. and thawed at room temperatures. The material was centrifuged at 3000 r.p.m. for 30 minutes. The supernatant fluid was drawn off and the residue was made up to the original volume with saline. The products were designated as Lots VII-C and VII-D, respectively.

3. Combined physical and chemical procedures.

a. Sonic oscillation - zinc precipitation. Lot VIII represents a 100-ml sample of starting vaccine which was treated in accordance with the method of Martin and Chapman (1954) after sonic vibrating for two minutes at approximately 10-k.c.

The sample of sonic-vibrated vaccine was zinc precipitated by adding zinc diglycinate reagent dropwise while gently stirring at 0°C. The reagent was added in the ratio of 0.25 ml per 5 ml of vaccine suspension. After the complete addition of the zinc reagent the mixture was adjusted to pH 7.4 with 1 N NaOH and allowed to come to equilibrium for one hour. The mixture was centrifuged at 3000 r.p.m. for 30 minutes and the supernate removed.

The precipitate was repeatedly extracted with 1 M glycine at pH 7.3. The extraction procedure was first carried out with a 100-ml

quantity of glycine and then with three successive 40-ml amounts. The mixture was centrifuged each time at 3000 r.p.m. for one hour at 3°C. The extracts were designated as Lots VIII-A, VIII-B, VIII-C and VIII-D, respectively. The residue of the final extraction was made up to its original volume with saline. This material was designated as Lot VIII-E.

b. Effect of sonic treatment on antigen solubility. A sample of the starting vaccine in saline, which was sonic vibrated for six minutes, was centrifuged for 30 minutes at 3500 r.p.m. The supernatant fluid was drawn off and designated Lot C-1.

The residue of the above sample was made up to its original volume with saline. This product was designated Lot C.

c. Ether extracted vaccine. An extraction procedure modified from that of Harris (1948) was followed, using ten volumes of ethyl ether as the extraction solvent. This was cooled to a temperature of about -50°C. and then the 6.66 per cent tissue vaccine at just above its freezing point was added dropwise with gentle stirring. Thereafter the mixture was brought to -10°C. over a period of several hours in a refrigerated bath. The ether was drawn off and the residue made up to volume with saline. This vaccine was then designated as Lot IX-A.

d. Ether-ethanol extracted vaccine. Ten volumes of an extraction solvent consisting of three parts ethyl ether and one part ethanol was employed in this extraction. The conditions employed were the same as those above. The residue vaccine was designated as Lot IX-B.

D. Antigenicity Testing

The potency (protective value) of the vaccine was determined in accordance with the Minimum Requirements for Rabies Vaccine of the National Institutes of Health (1953).

This test was based on the use of white Swiss mice approximately four weeks old, uniform in weight (11-15 gms) and of one sex.

Three or more dilutions of the vaccine under test were prepared using fivefold increments. The diluent employed was either saline or distilled water.

At least ten mice were injected intraperitoneally with 0.5 ml of each dilution of the vaccine. Two doses were given to each mouse one week apart. Enough control mice were set aside at the time of the injection of the first dose of vaccine, so that an adequate titration of the challenge virus could be made. At least ten mice were used for each dilution of challenge virus. The challenge virus was supplied by the National Institutes of Health.

At the time of challenge of the test mice, the control mice were divided into groups of at least ten mice and 0.03 ml of tenfold dilutions of the challenge virus injected intracerebrally. The dilutions were usually 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}. These control groups were inoculated with the challenge virus only after all the test mice had been inoculated.

All of the test mice were injected intracerebrally with 0.03 ml of a 10^{-6} dilution of the challenge virus 14 days after the first dose of vaccine.

This dilution of challenge virus usually gives challenge doses of 5 to 50 LD_{50}.

All mice were observed for 14 days from the time of the challenge injection. Only those deaths occurring after the fifth day were considered as rabies deaths. The mice which became paralyzed, but survived the 14-day observation period, were considered to have died with rabies.

Fifty per cent end-points were determined for the test vaccines and the controls by the method of Reed and Muench (1938). The end-points were calculated as an ED_{50} of vaccine in milligrams of original brain tissue which will protect 50 per cent of the mice. The LD_{50} of challenge virus received by the immunized mice is calculated by dividing the dilution of virus used as the test dose by the 50 per cent end-point dilution of virus in control mice as calculated by the method of Reed and Muench.

E. Chemical Analysis

The relative degree of rabies vaccine purification attained was based upon the reduction of protein nitrogen, total solids, and total ether-soluble lipids.

Protein nitrogen was determined by precipitation with 30 per cent trichloracetic acid followed by micro-Kjeldahl analysis of the nitrogen by a modification of the method of Ma and Zuazaga (1942).

Ether-soluble lipid was determined by continuous extraction in a micro-Soxhlet apparatus, following the procedure described in Methods of Analysis, Association of Official Agricultural Chemists.

Total solids were determined by drying samples to constant weight at 105° to 110°C.

IV. RESULTS

The application of the zinc precipitation and extraction procedure, advocated by Chapman and Surgenor for the purification of viruses, proved unsuccessful for the purification of rabies vaccine derived from rabbit brain. Only a small amount of the antigen of the original vaccine could be recovered in the purified form.

Failure to purify the vaccine without appreciably decreasing its antigenicity suggested that certain modifications of the method of precipitating and extracting the antigen should be made. These modifications were directed primarily at changing the hydrogen-ion concentrations.

The results of these alterations, as well as those of the original method, are shown in summary form in Table I. It will be noted that regardless of the pH of the zinc precipitating procedure or the treatment of the zinc-protein complex, only a small amount of antigen of the original vaccine could be obtained.

The zinc precipitation and extraction method of Martin and Chapman gave only slightly better results with respect to antigen yield. The results of the application of this method are shown in Table II. Inasmuch as the majority of the antigen of the starting vaccine can be accounted for in the various extractions, no measurable amount of antigen denaturation took place. This would indicate, then, that either the zinc failed to precipitate the majority of the antigen or that the glycine failed to efficiently extract the antigen from the zinc complex. In the repeated

TABLE I

pH MODIFICATIONS OF PURIFICATION PROCEDURE OF CHAPMAN AND SURGENOR

SUMMARY*

Lot	Antigenicity ED_{50}	Percent Total Antigen Recovery	pH		
			Precipitation Procedure	Zinc Acetate in Water Extraction	Zinc Acetate in Saline Extraction
Control	0.556 mg	--	---	---	---
I-A	3.50	16	7.4	5.6	5.6
Control	0.518	--	---	---	---
II-A	8.46	6	6.8	5.6	5.6
Control	0.880	--	---	---	---
III-A	3.78	23	7.4	5.8	6.1
III-B	6.24	14	7.4	5.6	5.8
III-C	5.56	16	7.4	5.8	5.9
Control	2.81	--	---	---	---
IV-A	25.0	11	7.8	5.5	5.5

*Original data presented in Appendix i

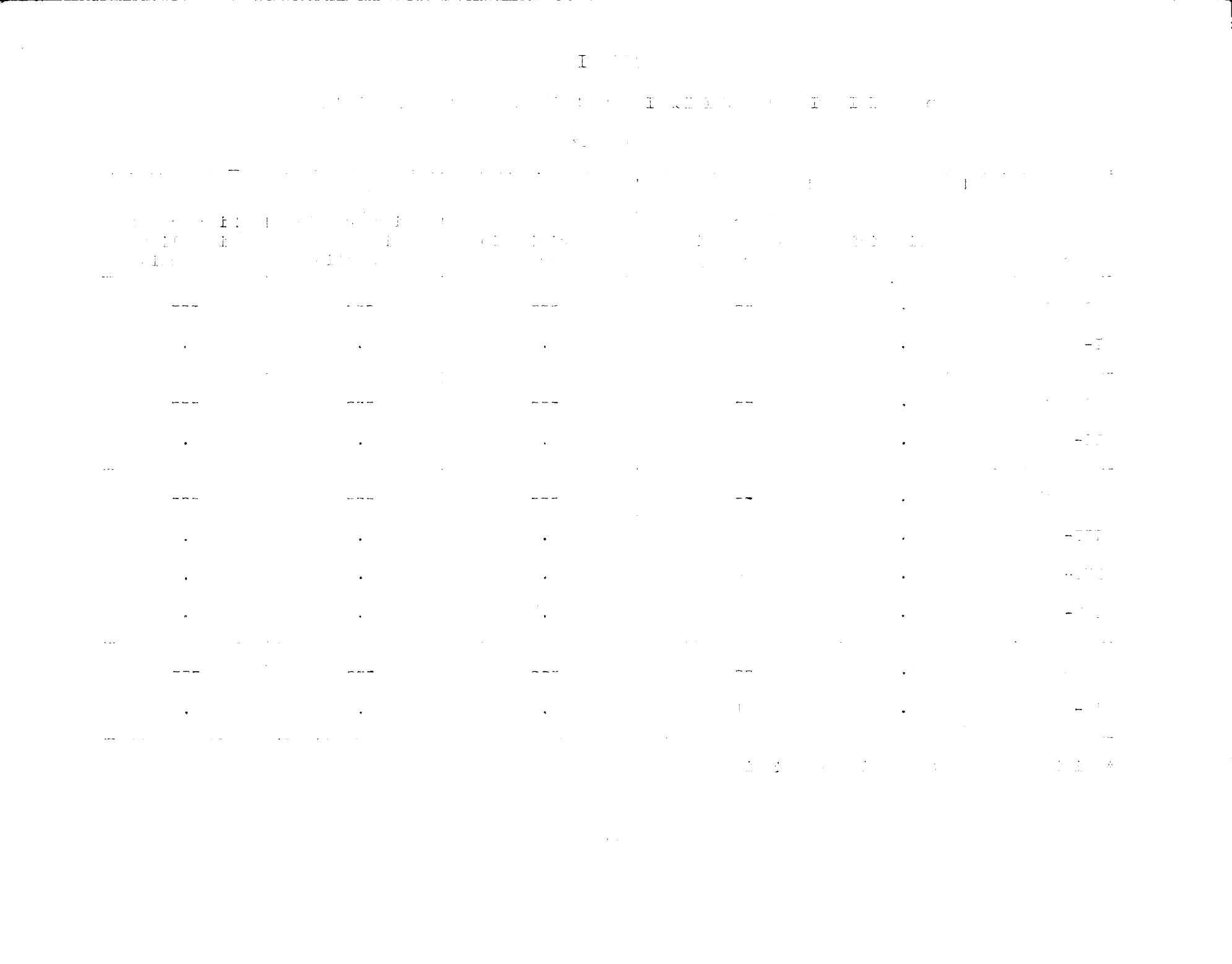

TABLE II

RESULTS OF PURIFICATION PROCEDURE OF MARTIN AND CHAPMAN

SUMMARY*

Lot	Antigenicity ED_{50}	Percent Total Antigen Recovery
Control	1.70 mg	--
Extract V-A	10.0	17
Extract V-B	10.0	17
Extract V-C	8.92	19
Extract V-D	>10.0	<17
Residue V-E	5.28	32.2

* Original data presented in Appendix ii

extractions of the zinc complex with glycine, substances which bind zinc least avidly are removed first, and those which bind zinc most strongly are removed last.

The results indicate, however, that most of the antigenic substance, if it were zinc precipitated, is not removed in any single extraction. If the zinc did not precipitate the major portion of the antigen, the glycine solutions merely extracted that portion of antigen which is soluble in the medium. The relatively constant amount of antigen obtained per extraction seems to indicate this, and that if the residue was again extracted, the residue would be reduced by a similar amount of antigen.

The zinc precipitating method was employed by others primarily for the precipitation and extraction of viruses from allantoic and amniotic

fluids and from aqueous and saline extracts of tissue. In view of this, it seems that the virus must be largely soluble or highly dispersed in the medium in order to be precipitated by zinc and subsequently extracted.

Brain tissue suspensions, which constitute the starting rabies vaccine, differ from allantoic fluid or aqueous and saline extracts of tissue in respect to solubility. It seems logical to assume that the solubility of the antigen plays an important role in the ability of the zinc to precipitate it.

Results of the study to determine the amount of antigen that can be expected to be present in the aqueous and saline extracts of rabies vaccine are shown in Table III. The extracts contained little antigen as compared to the entire vaccine. Approximately nine per cent of the total antigen was recovered in the aqueous extract. The saline extract of the vaccine, however, contained even less antigen. Approximately four per cent yield of antigen was obtained from the original vaccine in the saline extract.

These observations serve to point out the fact that since only a small amount of antigen is present in either the saline or aqueous extracts, it can be assumed that the majority of the antigenic component of rabies vaccine is associated with the small particles of brain tissue. Subsequently, this may indicate the reason for the apparent inability of the zinc to precipitate the bulk of the antigen from the suspension. The zinc is not effective in disrupting the cells or particles, but merely precipitates the material in solution.

The extraction of soluble antigen from tissue cells or particles, then, can only follow the further destruction of the cellular membranes, since these must be largely impermeable to the antigen.

TABLE III

STUDIES ON RABIES ANTIGEN SOLUBILITY

SUMMARY*

Lot	Antigenicity ED_{50}	Percent Total Antigen Recovery
Control A	0.50 mg	--
A-1 (Saline Extract)	12.60	4
Control B	0.292	--
B-1 (Aqueous Extract)	3.19	9
Control C	0.828	--
C-1 (Sonic treated Extract)	26.90	3

*Original data presented in Appendix iii

In view of the above observations, several methods of mechanical disruption of the cells or tissue particles were attempted on the starting brain tissue vaccine. The methods included slow and rapid freeze-thaw techniques and sonic oscillation.

The simplest and best method for the destruction of cellular membranes is considered to be disintegration by repeated freezing and thawing.

The effects of repeated freezing and thawing on the starting vaccine are shown in Table IV. Complete denaturation of the antigen, as measured by the mouse potency test, resulted from repeated slow freezing and thawing. Almost complete inactivation of the antigen was observed with the repeated rapid freeze and thaw technique.

TABLE IV

EFFECT OF REPEATED FREEZING AND THAWING ON RABIES VACCINE

SUMMARY*

Lot	Antigenicity ED_{50}	Antigen Recovery
Control	0.668	--
VI-A	Not measurable	0
VI-B	Not measurable	0
VI-C	Not measurable	0
VI-D	Not measurable	0

* Original data presented in Appendix iv

The effects of sonic oscillation, on the other hand, present quite a different picture. The results, shown in Table V, indicate the effect of sonic vibration on the antigenicity of the starting vaccine in relation to the time of exposure. It appears evident that sonic vibration for periods exceeding six minutes decreases the antigenicity of the vaccine appreciably. This is undoubtedly a denaturing effect which could be caused by mechanical agitation or thermal effects. However, sonic vibration for periods less than six minutes increased the antigenicity of the vaccine considerably. It appears reasonable to assume that the breaking up of the small particles or cells of tissue releases more antigen (virus), which results in a higher potency than would otherwise be attained with the vaccine.

TABLE V

EFFECT OF SONIC VIBRATION ON RABIES VACCINE

SUMMARY*

Lot	Time of Exposure in Minutes	Antigenicity ED_{50}	Relative Antigenicity ED_{50} Control Vaccine/ ED_{50} Test Vaccine
Control	--	1.07 mg	--
VII-2	2	0.564	1.90
VII-4	4	0.664	1.61
VII-6	6	0.604	1.77
VII-8	8	1.17	0.92
Control	--	1.18	--
VII-10	10	1.34	0.88
VII-14	14	1.39	0.85

*Original data presented in Appendix v

This observation suggested the possibility of obtaining a higher antigen yield from the starting vaccine, if the vaccine was first sonerated for the optimum time exposure before being precipitated by zinc. Inasmuch as the glycine extraction procedure appeared to be somewhat better than that of the Chapman and Surgenor method, the former method of extraction was employed.

As indicated in Table VI, the use of sonic oscillation in conjunction with zinc precipitation and repeated extractions with glycine increased the yield of purified vaccine to some extent. The yield represented an approximate twofold increase in the antigen recovered in the first glycine

extract. This amount of antigen, however, does not approach the minimum required to insure an adequate degree of protection. Further, subsequent extractions were not proportionately increased in antigen, indicating that increased solubility of the antigen had not necessarily been attained. The antigenicity of the residue decreased in direct proportion to the increase found in the first glycine extract. This may indicate that the further disintegration of the cells permits more antigen to be liberated in the form of an insoluble component which could not be precipitated from the non-sonerated vaccine due to its association with tissue cells. This does not mean that the antigen is rendered more soluble in the dispersing medium, but that it is made more available to the zinc.

TABLE VI

RESULTS OF PURIFICATION PROCEDURE OF MARTIN AND CHAPMAN ON SONIC VIBRATED RABIES VACCINE

SUMMARY*

Lot	Antigenicity ED_{50}	Percent Total Antigen Recovery
Control	1.70 mg	--
Extract VIII-A	4.92	34.6
Extract VIII-B	>10.0	<17.0
Extract VIII-C	7.88	21.6
Extract VIII-D	>10.0	<17.0
Residue VIII-E	10.0	17.0

*Original data presented in Appendix vi

On the basis of this observation, a further study on the solubility of the antigen was made. This study was conducted on a sonic vibrated sample of vaccine. The data of this study, also presented in Table III, indicate that the amount of soluble antigen present in the saline extract of the sonic vibrated sample was no greater than that present in the saline extract of the regular starting vaccine. Regardless of further disintegration of the cells by sonic oscillation, the solubility of the antigen remained relatively unchanged.

The results of attempts to free the starting rabies vaccine from the majority of the non-specific proteins and lipids of brain matter by employing ether and an ether-ethanol mixture as extractants are given in Table VII. No reduction in protein nitrogen was observed with either extractant. The ether extracted approximately 48 per cent of the lipids, and the ether-ethanol mixture extracted approximately 74 per cent. A comparable reduction in total solids was observed. Both techniques, however, greatly decreased the antigenicity of the vaccine.

These results indicate only that a mixture of ether and ethanol is more effective in removing the lipids from the vaccine than is ether alone. This may be due, in part, to the destructive action of ethanol on the cellular structures and/or the splitting of lipoprotein complexes by the ethanol.

Various chemical determinations (total nitrogen, total solids and total lipids) were performed on most of the purified vaccines studied. Since these vaccines were of insignificant antigenicity, the inclusion of these findings was not warranted.

TABLE VII

EFFECTS OF ETHER AND ETHER-ETHANOL EXTRACTION ON RABIES VACCINE

SUMMARY*

Lot	Antigenicity ED_{50}	Percent Total Antigen Recovery	Protein Nitrogen (mg/ml)	Total Solids Percent Dry Wt.	Percent Reduction Total Solids	Total Lipids Percent Dry Wt.	Percent Reduction Total Lipids
Control	1.10 mg	--	0.872	2.05	--	27.0	--
IX-A (Ether Extracted)	13.50	8	0.868	0.96	53.2	14.1	48
IX-B (Ether-Ethanol Extracted)	16.75	7	0.864	0.58	71.7	7.05	74

*Original data presented in Appendix vii

V. DISCUSSION

Chapman and Surgenor (1954) were successful in purifying viruses from allantoic and amniotic fluids and from aqueous and saline extracts of tissues. They employed a modification of the zinc precipitation method of Cohn (1953) for the purification of plasma proteins. A modification of this method was also used by Martin and Chapman (1954) for the purification of influenza virus from allantoic fluid.

These methods of virus purification, as well as modifications of the zinc precipitation methods, were applied without success in this study to the purification of rabies vaccine derived from rabbit brain.

In dispersing media, such as allantoic and amniotic fluids or extracts of tissue, the zinc precipitable substances apparently can be readily precipitated as zinc complexes. The precipitable substances can be regarded, therefore, as being largely soluble or highly dispersed in the medium. In any case, the substances are free to react with the zinc.

The small degree of solubility exhibited by the rabies antigen in aqueous and saline extracts of the vaccine indicates that the major portion of the antigen is associated with the minute tissue particles or the tissue cells. It was also observed that the antigenicity of the vaccine was enhanced when tissues were disintegrated by sonic oscillation. The saline extract of sonic treated vaccine, however, demonstrated no comparable increase in antigenicity, indicating the apparent insolubility of the antigen.

It was further observed that when sonic treated vaccine was zinc precipitated and extracted with glycine, more antigen was recovered than when this treatment was applied to the regular starting vaccine. This

indicated the possibility of having an insoluble antigen which is liberated, to some extent, from the brain cells or substances by sonic oscillation and subsequently precipitated by the zinc.

The action of the phenol, employed as the rabies virus inactivating agent, must also be considered in regard to the insolubility of the rabies antigen. The rabies virus may be complexed or denatured by the phenol or otherwise rendered more insoluble by its action.

Most denatured proteins are insoluble. However, some proteins can be rendered insoluble without any loss of biological activity. Johnson (1948) stated that live rabies virus is no better in invoking an immune response than an inactivated or killed virus. Inactivation of the virus, then, does not necessarily result in loss of antigenicity, although the inactivating agent could render it more insoluble. It may be pointed out, however, that some workers were able to maintain the Flury strain of living rabies virus in chick embryos and were able to induce biological and immunological changes that were significant contributions to the preparation of a commercial vaccine of high antigenicity.

It has been observed that a large portion of the live rabies virus is found in the sediment of macerated brain tissue as shown by regrinding the sediment and titrating the supernate. The virus particle may combine with and remain fixed to host tissue components as do certain other virus particles (Curnen and Horsfall, 1946). Similarly, the antigenicity of phenol-inactivated rabies vaccine is largely found in the sediment.

Attempts to purify the rabies vaccine by zinc precipitation of the antigen followed by selective dissociation of the zinc complex was apparently accomplished only on that portion of the antigenic component

which can be considered to be free to react with the zinc. The zinc is probably not effective in disrupting the tissue particles or the chemical complexes which may have been formed with the phenol.

Whereas many proteins remain in the native state when their solutions are frozen and thawed repeatedly, the solubility and biological properties of lipoproteins are affected by such treatment. It is well known that the lipoproteins of plasma are denatured upon repeated freezing and thawing (McFarlane 1942, Haurowitz 1950). Johnson (1948) stated that repeated freezing and thawing of live rabies virus suspensions results in the loss of infectivity. It was also observed in this study that the antigenic component of rabies vaccine or the inactivated virus was completely destroyed by repeated freezing and thawing.

A considerable portion of the lipids of rabies vaccine cannot be extracted by treatment with ether. This may be due to the presence of lipoprotein complexes. The lipoproteins are, however, split by the action of ethanol. Inasmuch as the temperatures of the ether and ethanol extractions employed in this study never exceeded -10°C, it was felt that thermal denaturation of the rabies antigen was not indicated. However, when ether was employed as the extraction agent for the removal of lipids from rabies vaccine, almost complete loss of antigen resulted. A similar loss of antigen resulted from the use of an ether-ethanol mixture, although the amount of lipid extracted was considerably greater. This only indicates that the ether-ethanol mixture is a better lipid extracting agent than ether alone. However, there is the possibility that the use of ether at -50°C. may have partially denatured the rabies antigen as was

shown with the repeated freeze and thaw technique. McFarlane (1942) employed a method of freezing and thawing with ether to split lipoproteins.

Some purified viruses have been found to contain significant amounts of lipid. Equine encephalomyelitis virus was found to contain large amounts of lipid in the form of phospholipid, cholesterol and neutral fat (Beard, 1945). Influenza virus was found to contain lipid, in addition to nucleoprotein (Knight, 1947). Although data on the chemical nature of the rabies virus are not available, it is possible that this virus also contains lipids and that the action of lipid solvents denatures the virus. It has been shown that the rabies virus is only moderately resistant to ether, but very resistant to phenol (Johnson, 1948).

In addition to the denaturation of the rabies antigen and virus by lipid solvents, it has been shown that the rabies antigen, as well as the active virus, is denatured by repeated freezing and thawing. This phenomenon is characteristic of lipoproteins. It is also possible that lipids, in some manner, may protect the rabies antigen or virus in some chemical combination.

It is the author's opinion that before any acceptable method of rabies vaccine purification can be accomplished, it will be necessary first to characterize chemically the inactivated rabies virus. The intimate association of the antigen with brain substances or protective chemical complexes has defied most physical and chemical methods of separation without denaturation of the antigen.

VI. SUMMARY

The zinc precipitation and extraction procedures advocated by Chapman and Surgenor (1954) and Martin and Chapman (1954) for the purification of certain viruses were unsuccessful when applied to the purification of rabies vaccine derived from rabbit brain. Modifications of the method likewise proved unsatisfactory. Although attempts to purify the rabies vaccine without appreciably decreasing its antigenicity failed, significant information was obtained concerning the antigen.

The rabies antigen is largely insoluble or poorly dispersed in aqueous and saline solutions. This is due either to the intimate association of the antigen with the brain substances or to its chemical composition. This insolubility is thought to be the primary cause for the failure of the zinc to precipitate the major portion of the antigen from the vaccine.

Sonic oscillation for short intervals greatly increases the antigenicity of the rabies vaccine. However, no comparable increase is found in its antigen solubility. The liberation of some insoluble antigen, which was zinc precipitable, follows sonic disintegration of the tissue cells.

Repeated freezing and thawing of rabies vaccine results in the complete destruction of the antigenic component. Similarly, the extraction of the vaccine with ether or a mixture of ether and ethanol at low temperatures results in a great loss of antigenicity. The similarity of the rabies antigen to lipid-protein combinations is discussed.

It is concluded that the chemical characteristics of the inactivated rabies virus must first be more adequately determined before an acceptable method of rabies vaccine purification can be developed.

VII. LITERATURE CITED

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Appendix i

Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed totals		Percent Mortality	ED_{50}
				S	D		
I Control	1:6.66	10.0	17/18	43	1	23	0.556 mg
	1:33.3	2.0	12/18	24	7		
	1:166.5	0.4	9/18	12	16	57	
	1:832.5	0.08	3/18	3	31		
I-A	1:6.66	10.0	12/18	21	6	65	3.50 mg
	1:33.3	2.0	7/18	9	17		
	1:166.5	0.4	2/16	2	33		
	1:832.5	0.08	0/18	0	51		

Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals		Percent Mortality
		S	D	
10^{-6}	1/12	1	19	50
10^{-7}	7/12	8	8	
10^{-8}	9/12	17	3	
10^{-9}	12/12	29	0	

Challenge dose of virus = 10.0 LD_{50}

Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals		Percent Mortality	ED_{50}
				S	D		
II Control	1:33.3	2.0	17/18	26	1	3	
	1:166.5	0.4	6/18	9	13	59	0.518 mg
	1:832.5	0.08	3/18	3	28		
II-A	1:6.66	10.0	6/20	16	14	47	
	1:33.3	2.0	3/20	10	31	76	8.46 mg
	1:166.5	0.4	6/19	7	44		
	1:832.5	0.08	1/20	1	63		

Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals		Percent Mortality
		S	D	
10^{-6}	0/12	0	19	
10^{-7}	5/12	5	7	57
10^{-8}	12/12	17	0	0

Challenge dose of virus = 13.3 LD_{50}

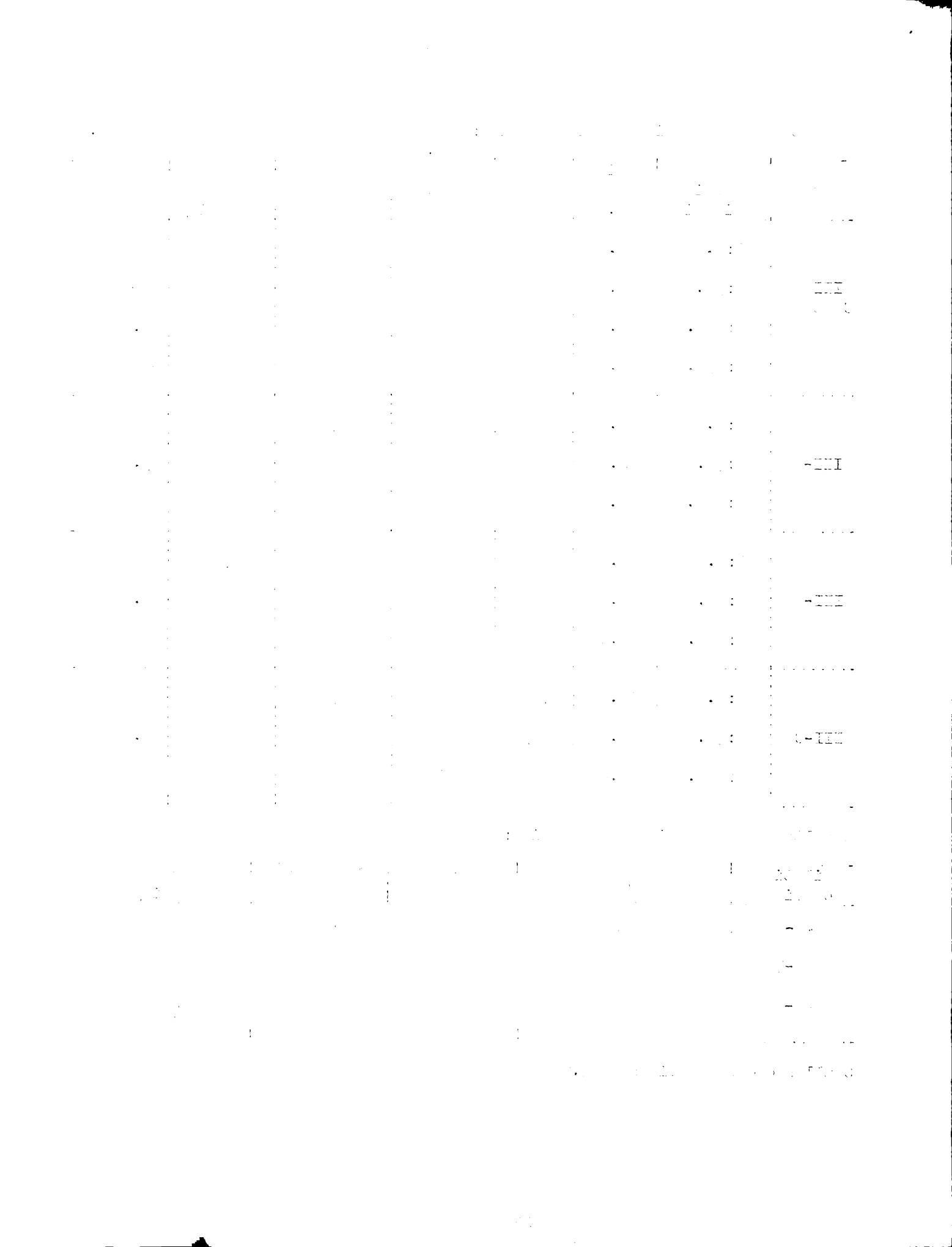
Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality	ED_{50}
III Control	1:6.66	10.0	17/18	35	1		
	1:33.3	2.0	14/17	18	4	18	
	1:166.5	0.4	4/17	4	17	81	0.880 mg
	1:832.5	0.08	0/18	0	35		
III-A	1:6.66	10.0	7/10	11	3	21	
	1:33.3	2.0	4/10	4	9	69	3.78 mg
	1:166.5	0.4	0/10	0	19		
III-B	1:6.66	10.0	2/8	8	6	43	
	1:33.3	2.0	4/10	6	12	67	6.24 mg
	1:166.5	0.4	2/10	2	20		
III-C	1:6.66	10.0	7/10	9	3	30	
	1:33.3	2.0	1/9	2	11	85	5.56 mg
	1:166.5	0.4	1/10	1	20		

Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality
10^{-6}	0/12	0	24	
10^{-7}	2/12	2	12	86
10^{-8}	10/12	12	2	17

Challenge dose of virus = 33.2 LD_{50}



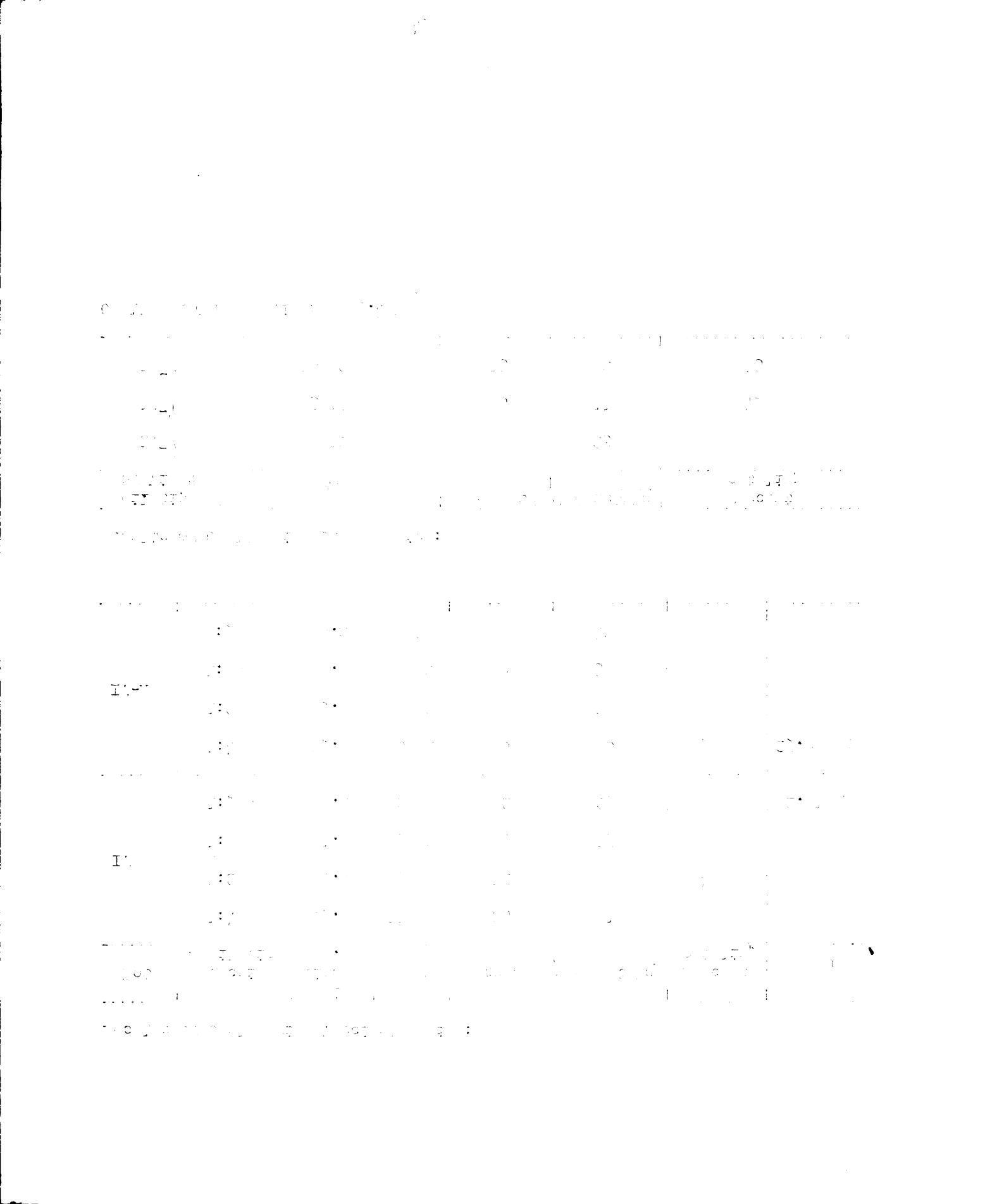
Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality	ED_{50}
IV	1:4	25.0	11/12	23	1		
	1:20	5.0	5/12	12	8	40	
	1:100	1.0	5/12	7	15	68	
	1:500	0.2	2/12	2	25		2.81 mg
IV-A	1:4	25.0	6/12	6	6	50	25.0 mg
	1:20	5.0	0/12	0	18		
	1:100	1.0	0/12	0	30		
	1:500	0.2	0/12	0	42		

Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality
10^{-6}	0/12	0	23	
10^{-7}	3/12	3	11	79
10^{-8}	10/12	13	2	13

Challenge dose of virus = 27.4 LD_{50}



Appendix ii

Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals		Percent Mortality	ED_{50}
				S	D		
V Control	1:6.66	10.0	12/12	18	0		
	1:33.3	2.0	5/10	6	5	45	
	1:166.5	0.4	1/12	1	16	94	1.70 mg
V-A	1:6.66	10.0	6/12	6	6	50	10.0
	1:33.3	2.0	0/12	0	18		
	1:166.5	0.4	0/10	0	28		
V-B	1:6.66	10.0	3/11	8	8	50	10.0
	1:33.3	2.0	3/11	5	16		
	1:166.5	0.4	2/12	2	26		
V-C	1:6.66	10.0	6/12	7	6	46	
	1:33.3	2.0	1/12	1	17	94	8.92
	1:166.5	0.4	0/11	0	28		
V-D	1:6.66	10.0	4/11	5	7	58	>10.0
	1:33.3	2.0	1/12	1	18		
	1:166.5	0.4	0/11	0	29		
V-E	1:6.66	10.0	6/11	10	5	33	
	1:33.3	2.0	3/11	4	13	76	5.28
	1:166.5	0.4	1/12	1	24		

Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals		Percent Mortality
		S	D	
10^{-6}	0/12	0	22	
10^{-7}	2/12	2	10	83
10^{-8}	11/11	13	0	0

Challenge dose of virus = 25.0 LD_{50}

Appendix iii

Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals		Percent Mortality	ED_{50}
				S	D		
A Control	1:6.66	10.0	13/13	30	0		
	1:33.3	2.0	11/12	17	1	6	
	1:166.5	0.4	6/13	6	8	57	0.50 mg
A-1	Undiluted	66.6	10/13	17	3	15	
	1:6.66	10.0	6/12	7	9	56	12.60 mg
	1:33.3	2.0	1/13	1	21		

Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals		Percent Mortality
		S	D	
10^{-6}	0/12	0	21	
10^{-7}	4/12	4	9	69
10^{-8}	11/12	15	1	6
10^{-9}	12/12	27	0	

Challenge dose of virus = 20.0 LD_{50}

Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality	ED_{50}
B Control	1:100	1.0	16/23	31	7	18	
	1:500	0.2	7/23	15	23	60	0.292 mg
	1:2500	0.04	8/22	8	37		
B-1	1:20	5.0	11/22	18	11	38	
	1:100	1.0	4/23	7	30	81	3.19 mg
	1:500	0.2	2/23	3	51		
	1:2500	0.04	1/23	1	73		

Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality
10^{-6}	1/12	1	17	94
10^{-7}	8/12	9	6	40
10^{-8}	10/12	19	2	
10^{-9}	12/12	31	0	

Challenge dose of virus = 6.51 LD_{50}

Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals		Percent Mortality	ED_{50}
				S	D		
C Control	1:6.66	10.0	10/12	24	2		
	1:33.3	2.0	10/12	14	4	22	
	1:166.5	0.4	4/11	4	11	73	0.828 mg
	1:832.5	0.08	0/12	0	23		
C-1	Undiluted	66.6	9/12	12	3	20	
	1:6.66	10.0	2/13	3	14	82	26.9 mg
	1:33.3	2.0	1/13	1	26		
	1:166.5	0.4	0/13	0	39		

Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals		Percent Mortality
		S	D	
10^{-6}	0/12	0	21	
10^{-7}	4/12	4	9	69
10^{-8}	11/12	15	1	6
10^{-9}	12/12	27	0	

Challenge dose of virus = 20.0 LD_{50}

Appendix iv

Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality	ED_{50}
VI Control	1:6.66	10.0	15/15	35	0		
	1:33.3	2.0	15/16	20	1	5	
	1:166.5	0.4	5/16	5	12	71	0.668 mg
VI-A	1:6.66	10.0	0/16				
VI-B	Undiluted	66.6	6/15				
	1:6.66	10.0	1/14				
	1:33.3	2.0	0/16				
VI-C	Undiluted	66.6	0/12				
VI-D	Undiluted	66.6	0/12				
	1:6.66	10.0	0/11				

Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality
10^{-6}	0/12	0	21	
10^{-7}	5/12	5	9	64
10^{-8}	10/12	15	2	12

Challenge dose of virus = 18.6 LD_{50}

Appendix v

Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals		Percent Mortality	ED_{50}
				S	D		
VII Control	1:6.66	10.0	11/11	20	0		
	1:33.3	2.0	6/10	9	4	31	
	1:166.5	0.4	3/11	3	12	80	1.07 mg
VII-2	1:6.66	10.0	10/10	24	0		
	1:33.3	2.0	8/10	14	2	13	
	1:166.5	0.4	3/10	6	9	60	0.564 mg
	1:832.5	0.08	3/10	3	16		
VII-4	1:6.66	10.0	12/12	25	0		
	1:33.3	2.0	9/11	13	2	13	
	1:166.5	0.4	4/10	4	8	67	0.664 mg
	1:832.5	0.08	0/11	0	19		
VII-6	1:6.66	10.0	8/10	24	2		
	1:33.3	2.0	9/12	16	5	24	
	1:166.5	0.4	6/11	7	10	59	0.604 mg
	1:832.5	0.08	1/12	1	21		
VII-8	1:6.66	10.0	10/10	19	0		
	1:33.3	2.0	6/11	9	5	36	
	1:166.5	0.4	3/9	3	11	78	1.17 mg
	1:832.5	0.08	0/12	0	23		

Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality
10^{-6}	0/11	0	19	
10^{-7}	4/12	4	8	67
10^{-8}	11/11	15	0	0

Challenge dose of virus = 18.0 LD_{50}

Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality	ED_{50}
VII Control	1:6.66	10.0	16/16	34	0		
	1:33.3	2.0	15/16	18	1	6	
	1:166.5	0.4	3/16	3	14	82	1.18 mg
VII-10	1:6.66	10.0	12/16	26	4		
	1:33.3	2.0	11/16	14	9	39	
	1:166.5	0.4	3/16	4	21	84	1.34 mg
	1:832.5	0.08	1/16	1	36		
VII-14	1:6.66	10.0	13/16	26	3		
	1:33.3	2.0	11/16	13	8	38	
	1:166.5	0.4	2/16	2	22	92	1.39 mg

Results of Challenging Control Mice:

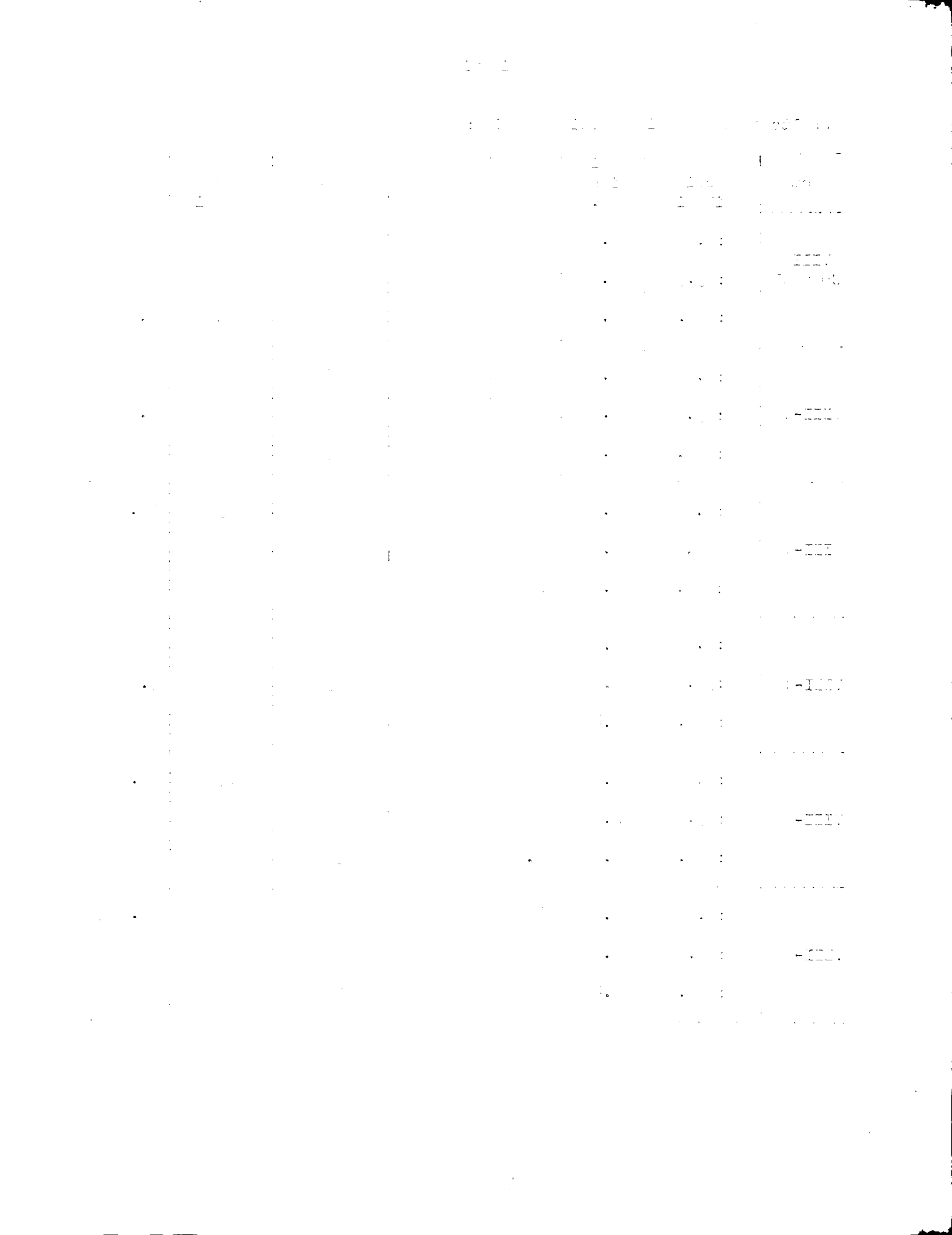
Dilution of Virus	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality
10^{-6}	0/12	0	22	
10^{-7}	5/12	5	10	67
10^{-8}	9/12	14	3	18

Challenge dose of virus = 22.2 LD_{50}

Appendix vi

Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals		Percent Mortality	ED_{50}
				S	D		
VIII Control	1:6.66	10.0	12/12	18	0		
	1:33.3	2.0	5/10	6	5	45	
	1:166.5	0.4	1/12	1	16	94	1.70 mg
VIII-A	1:6.66	10.0	8/9	8	1	11	
	1:33.3	2.0	0/11	0	12	100	4.92 mg
	1:166.5	0.4	0/11	0	23		
VIII-B	1:6.66	10.0	3/12	8	9	53	10.0 mg
	1:33.3	2.0	3/11	5	17		
	1:166.5	0.4	2/11	2	26		
VIII-C	1:6.66	10.0	3/11	9	8	47	
	1:33.3	2.0	5/10	6	13	68	7.88 mg
	1:166.5	0.4	1/12	1	24		
VIII-D	1:6.66	10.0	3/12	4	8	67	10.0 mg
	1:33.3	2.0	1/12	1	19		
	1:166.5	0.4	0/12	0	31		
VIII-E	1:6.66	10.0	4/12	8	8	50	10.0 mg
	1:33.3	2.0	2/12	4	18		
	1:166.5	0.4	2/12	2	28		



Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals		Percent Mortality
		S	D	
10^{-6}	0/12	0	22	
10^{-7}	2/12	2	10	83
10^{-8}	11/11	13	0	0

Challenge dose of virus = 25.0 LD_{50}

Appendix vii

Results of Challenging Vaccinated Mice:

Lot	Vaccine Dilution	Brain tissue mg.	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality	ED_{50}
IX Control	1:6.66	10.0	13/16	29	3		
	1:33.3	2.0	11/16	16	8	33	
	1:166.5	0.4	5/16	5	19	79	1.10 mg
IX-A	Undiluted	33.3	7/16	15	9	37	
	1:6.66	5.0	5/16	8	20	71	13.50 mg
	1:33.3	1.0	3/16	3	33		
	1:166.5	0.2	0/16	0	49		
IX-B	Undiluted	33.3	7/16	13	9	41	
	1:6.66	5.0	5/16	6	20	77	16.75 mg
	1:33.3	1.0	0/16	1	20		
	1:166.5	0.2	1/16	1	21		

Results of Challenging Control Mice:

Dilution of Virus	S/T	Reconstructed Totals S	Reconstructed Totals D	Percent Mortality
10^{-6}	0/12	0	25	
10^{-7}	1/12	1	13	93
10^{-8}	10/12	11	2	15

Challenge dose of virus = 35.6 LD_{50}

Appendix viii

Zinc Reagent

0.25 M glycine

0.1 M zinc oxide

0.4 M zinc acetate

To 125 ml hot distilled water 18.77 gm glycine was dissolved. The mixture was heated but not boiled. The mixture was stirred constantly and 8.14 gm zinc oxide dusted into the hot glycine. The zinc oxide was added slowly and in small portions so that it goes completely into solution before another aliquot was added. In this way the complete conversion to zinc diglycinate was obtained and the resultant solution was clear.

To 300 ml distilled water 87.8 gm zinc acetate ($Zn(C_2H_3O_2)_2 \cdot 2H_2O$) was dissolved. This solution was added to the zinc diglycinate prepared above. The final volume was adjusted to 1 liter with distilled water. The solution was tightly stoppered and refrigerated.

Date Due

NOV 1 '65			