LOYD WAYNE TIFFANY Ph. D. 1955

A PARTIAL AND COMPARATIVE EVALUATION IN MICE OF AN ALUMINUM PHOSPHATE ADSORBED ANTI-SWINE ERYSIPELAS BACTERIN PREPARED BY THE SONIC BACTERIOCLASIS OF ERYSIPELOTHRIX RHUSIOPATHIAE

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
Loyd Wayne Tiffany
1955

This is to certify that the

thesis entitled

"A Partial and Comparative Evaluation in Mice of an Aluminum Phosphate Adsorbed Anti-Swine Erysipelas Bacterin Prepared by the Sonic Bacterioclasis of Erysipelothrix Rhusiopathiae"

presented by

Loyd W. Tiffany

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Microbiology

W. J. Stafseth
Major professor

Date October 31, 1955

O-169

A PARTIAL AND COMPARATIVE EVALUATION IN MICE OF AN ALUMINUM PHOSPHATE ADSORBED ANTI-SWINE ERYSIPELAS BACTERIN PREPARED BY THE SONIC BACTERIOCLASIS OF ERYSIPELOTHRIX RHUSIOPATHIAE

Loyd Wayne Tiffany

A THESIS

Submitted to the School of Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1955

ACKNOWLEDGEMENT

The author wishes sincerely to acknowledge the direction, counsel, and assistance afforded to this work and person by Dr. J.J. Stockton. Further, the author wishes to express his gratitude to Dr. R.D. Barner for his interest in, and support of, this problem. Assistance rendered by Mr. W. Gebhard and Dr. R. Dresens of the Michigan Department of Health also commands a large portion of the author's recognition.

In all sincerity, acknowledgement is to be made to the many others who have aided the author in lesser roles, and who, though anonymous in this acknowledgement, merit and receive the author's most candid appreciation.

Loyd Wayne Tiffany

AN ABSTRACT

of

A Partial and Comparative Evaluation in Mice of an Aluminum Phosphate Adsorbed Anti-Swine Erysipelas Bacterin Prepared by the Sonic Bacterioclasis of Erysipelothrix rhusiopathiae.

Attention was directed to an apparent increase in swine erysipelas and to the economic loss which accompanied this infection. The need of more acceptable prophylactic and therapeutic measures for controlling this disease was stressed. Procedures for challenging experimental animals and methods of prophylaxis available at the present time have been discussed.

The object of this problem was the production and evaluation of a bacterin which consisted of formalin killed, sonically disrupted cells of Erysipelothrix rhusiopathiae adsorbed onto the surface of aluminum phosphate particles.

Three strains of E. rhusiopathiae were selected for the production of bacterins. The cells were killed by the addition of 0.3 per cent formalin to the cultures and were separated from the medium by centrifugation. These cells were then disrupted by sonic energy and adsorbed onto aluminum phosphate. The medium used for cultivation of the cells was also adsorbed with aluminum phosphate and the adsorbate products combined with the cell adsorbate. White mice were used to test these products against a challenge of undiluted virulent culture and against a challenge of 300LD_{50}.

Strain 14774 did not protect any of the test animals and strains SE-9 and SE-25 protected 100 per cent of the animals, injected with the undiluted bacterin, against the challenge of undiluted culture. Against

a challenge of $300LD_{50}$, bacterin SE-9 protected 100 per cent of the animals in a dilution of 1-4 and SE-25 protected 100 per cent of the animals in a dilution of 1-2 against a similar challenge.

A commercial bacterin was evaluated for the purpose of comparison and proved to offer greater protection than either of the experimental products. It protected 100 per cent of the animals in a dilution of 1-2 against a challenge with undiluted culture and 100 per cent of the animals were protected against a challenge of $300LD_{50}$ in a dilution of 1-8 of the commercial bacterin.

Considering the variables present, for which more refined evaluation is indicated, the most important development of this work was the promise which this procedure offers for the development of an acceptable bacterin.

TABLE OF CONTENTS

Introduction

Since the beginning of organized research relative to swine erysipelas (about 1880), a considerable amount of knowledge has been accummulated regarding the nature and control of the causative organism, Erysipelothrix rhusiopathiae. However, though many important characteristics of this organism and its associated disease have been clearly elucidated, a satisfactory understanding of the facts sufficient for the establishment of an adequate control program remains, as yet, obscure.

Kaplan (1948) has estimated that the economic loss due to swine erysipelas exceeds that of hog cholera in Europe, and Ray (1952) has reported it to be the second most prevalent disease of swine in the United States. Ray (1952) reported that in 1951 this disease was prevalent in 22 states and that 17 states reported an increase in the range of occurrence of this infection. Cooper (1954) has reported that the prevalence of infection in turkeys has increased to epizootic proportions in several states of the western United States, and reports by Marsh (1931), Breed (1938), and Ray (1930), attest the losses inflicted on the sheep industry by this disease entity.

Also to be acknowledged are reports of vaccine erysipelas, Fluckiger (1949, 1952, 1952); i.e. cases of this disease which occur after vaccination with live culture and antiserum and which are probably a result of the inoculation.

The actual incidence of swine erysipelas in Michigan is thought to be very low, but evidence is accummulating which suggests an increase at least in the number of recognized cases of

swine erysipelas infection within this state Veterinary Reporting Service (1954). For the most part, the chronic form of this disease has been predominant in Michigan; however, during the past year several acute cases have been observed of which a number have resulted in death losses.

Several methods of control have been developed which have been very promising in their early evaluations. The simultaneous method of administration of antiserum and live culture, the inception of the use of formalin killed, aluminum hydroxide adsorbed bacterin and recently, the lysebacterin, the use of antibiotics both separately and mixed with antiserum, and vaccines consisting of avirulent strains of E. rhusiopathiae have been developed. Each has been accompanied by rather disappointing limitations which were revealed by repeated field and laboratory applications of these products.

Continued, and perhaps increased, economic losses due to swine erysipelas serve as a stimulus to more intensive investigational efforts regarding control and prevention of this infection. Concern is justly directed to the lack of satisfactory control measures and the continued appearance of the infection in the animal and bird populations. The continued demand on the swine, sheep, and turkey industries for human sustenance in itself emphasizes the pressing need for more acceptable methods of understanding and control.

The present investigation, of which this thesis is a report, was undertaken in order to determine the value, in mice, of a bacterin prepared by the sonic disruption of E. rhusiopathiae for protection against a challenge dose of this same organism. A number of techniques and numerous bits of information have been adopted

from the available literature and utilized in the production of the experimental bacterins and, though the nature of the problem invites a more prolonged investigation, the limitations of time and economics permit only a brief examination of this one greatly delimited phase.

Review of the Literature

Prior to the great swine plague, the abstruse nature of swine erysipelas is evidenced by the numerous colloquial synonyms which were applied to this infection. Swine typhus, wild fire, the rose, St. Anthony's fire, spotted fever and spotted fire were some of the commoner terms used to describe a syndrome, the chief characteristic of which was an erythematous condition of the skin. No doubt, the nature of the symptoms was the great motivating force which resulted in the lumping together of several distinct diseases under one or more of the mentioned terms. The term "swine erysipelas" was no exception to this practice since it was also applied at times to diseases which were accompanied by redness of the skin. However, a rather inclusive examination of the old literature by Friedberger and Frohner (1910) revealed that the term "swine erysipelas" usually referred to the disease, swine erysipelas, as we recognize it today. This would indicate that swine erysipelas as such has been a more or less covert disease in Europe for many centuries.

The great swine plague of hog cholera which in 1877-78 swept the United States, England, the Scandanavian countries and most of Europe, probably provided an impetus to the early investigation of swine diseases.

Subsequently, Robert Koch (1880) isolated an organism from a mouse which he had previously injected with a quantity of putrefying blood. He named this organism Erysipelothrix muriseptica. However, Koch was not known to have associated this organism with swine erysipelas.

Following Koch's work, Thuillier in 1882, Pasteur and Thuillier

(1883), isolated an organism from pigs dying of rouget. This organism was poorly described by Pasteur and Dumas (1883).

About four months after Thuillier's observation, Loeffler (1885) cultured an organism from the blood vessels of the skin of a pig which had died of swine erysipelas. He gave a more accurate and inclusive description of the etiologic agent and the clinical manifestations of swine erysipelas than did his contemporaries, and though he did not publish his findings until 1885, received the credit for describing this organism for the first time and associating it with swine erysipelas.

Pasteur and Thuillier (1883) observed the existence of an inverse relationship between the virulence of the swine erysipelas bacillus in rabbits and pigs. As the organism increased in virulence for the rabbit, it decreased in virulence for the pig. This observation was the basis for the first vaccine against swine erysipelas. The modified attenuated culture was administered to pigs and followed twelve days later by an injection of a culture which had been enhanced in virulence by passage through pigeons. Since this was the only prophylactic procedure available at that time, it was adopted and used rather extensively in Europe. Forthcoming from the practical evaluations of this vaccine, were numerous objections based, in part, on the anxiety created by the use of live culture as a vaccine. The period of actual protection was questionable and a suitable means of challenging experimental animals was not at their disposal. Thus the search for a more acceptable control agent continued.

A second phase of the historical investigation of swine erysi-

pelas control agents began with the observations of Emmerich and Mastbaum (1891), who demonstrated the protection afforded by the blood serum of rabbits which had been immunized against the swine erysipelas bacillus. Subsequently, Lorenz, (1893, 1894, 1896) Leclainche (1897) and Schutz and Voges (1898) began to experiment using culture and antiserum and in 1896, Lorenz published a description of a "Simultaneous Method" of immunization against swine erysipelas. This method consisted of the injection of live virulent culture simultaneously with a specific antiserum. This procedure was supplemented by a second injection of virulent culture only, about 14 days following the primary injection if a more prolonged immunity was desired. Leclainche (1897) also offered a method which was very similar to that proposed by Lorenz. The methods differ in that the Leclainche procedure requires the vaccine and antiserum to be mixed prior to injection, and followed in 10-12 days by a second administration of vaccine alone. The Lorenz method necessitated the injection of antiserum and culture simultaneously into different parts of the body. The "simultaneous method" of immunization against swine erysipelas has undergone numerous field and laboratory evaluations and though its use is still limited in the United States and certain other countries, remains one of the most effective control procedures available at the present time. For the most part, the limitations imposed on this technique are based on an anxiety created by the necessity of using live culture to combat the infection, since this involves the possible spread of the disease and poses a potential hazard to the person administering the vaccine. Further, the economics involved in the production of the antiserum calls for a more desirable method.

Live culture conferring only a relatively short and uncertain duration of protection, plus the economics involved in the production of antisera, are the main objections to the all-out acceptance of this technique. However, these objections of themselves serve to emphasize the need of better control products. The existent anxiety may, in part, be unjust, in that too much may be expected from an otherwise effective control product. This product, if properly utilized, is a powerful supplement to the present poorly-stocked arsenal of control procedures. In areas of known soil contamination e.g., one could hardly prohibit the use of this vaccine technique since it is one of the most successful methods at one's present disposal. However, in areas where there has been little history of the disease, one would be justified in exercising caution in introducing such a procedure.

Muromcev and Matvyenko (1934), in attempting to evaluate the effects created by the changes in density, surface tension, and osmotic pressure, brought about by the addition of varying amounts of agar to liquid medium, felt that this addition of agar created an environment in vitro which more closely approximated that present in the animal body than did a solid or broth medium. They found that E. rhusiopathiae grew very well in their "agaro-bullion" and thus they observed a basis for the production of a vaccine against swine erysipelas. Probably attempting to circumvent the objections to the use of live culture vaccines, they produced a killed bacterin through the addition of 0.3 per cent formalin to the cultures of E. rhusiopathiae. The promise of such an agent was quickly recognized and after preliminary investigations of the vaccine in 1934 and 1935,

large scale inoculations of the Muromcev vaccine were carried out in 1936 and 1938 (Jastrzebski 1945). Again, a means of challenge of swine, supposedly protected by this vaccine, was not available and results were confined to field observations of the incidence of infection. Also, control animals were few and not necessarily located in the same herd for which they were to be the standard. Though much promise was evidenced from these observations, the lack of the original Russian literature and the existent world conditions at that time necessitated a further evaluation, which was accomplished with the aid of a more recent technique. This vaccine was not abandoned, however, as Kaplan (1948) reported on extensive trials of this vaccine in Poland. Some aspects of the procedure (preparation of the vaccine) have doubtless influenced other more recent developments in the field of erysipelas prophylaxis.

Kondo and Sugimura (1935) attempted to produce a vaccine against swine erysipelas by culturing the organism in increasing amounts of trypaflavin and, though the organisms lost their pathogenicity for mice, they regained it after culturing on agar. This was a step toward the selection of a culture of very low or lacking in virulence for swine.

Staub (1940) isolated a strain of E. rhusiopathiae which appeared to be an avirulent mutant. This strain was used quite extensively in France and in the French colonies as well as in Poland, and, though some authors reported success, there were not enough reports of experiments with controls to prove the value of this vaccine. Staub maintains that the strain is practically avirulent for mice but Gladhill (1949) was able to demonstrate virulence for

mice after several passages in serum broth.

Fortner (1944) provided a significant advance toward the evaluation of the level of immunity induced by various vaccine preparations through the introduction of the "Scarification Technique" for challenge. Prior to the inception of this technique, the knowledge of levels of immunity attained by vaccines was based primarily on gross field and laboratory observations of control and immunized animals. No satisfactory means of determining the degree of protection conferred by various vaccine products or the susceptability of unexposed animals existed. The advent of this technique was timely and welcome. Fortner (1946, 1947) was able to demonstrate that little or no immunity was produced in response to the formalin killed vaccine of Muromcev (1934). Since this method constitutes a severe challenge and necessitates the implication of a number of animals, (see description of method below) it stands as the most acceptable means of evaluation of immunity level available today.

This method consisted of the production of parallel scarifications on the lateral surface of the thorax and abdomen of a number of swine (Fortner used eight swine) not deep enough to induce hemorrhage, but of sufficient depth as to yield an effusion of tissue fluid. A culture to be tested was then rubbed into one line on each of the pigs and the response noted. The response may be local edema along the line of scarification, accompanied by a rise in temperature, or a generalized septicemia or chronic arthritis may result.

Since this method of Fortner did not lend itself to the preciseness desirable in laboratory procedures, Hars and Delpy (1953) developed a method which consisted of the titration of strains with

antiserum in the pig skin by means of intradermal inoculations. Hars's and Delpy's experiments demonstrate a significant value for this test though it does involve the use of antiserum which is of economic importance.

Following the introduction of the scarification technique developed by Fortner (1944), a means was then available for the evaluation of existing and subsequent vaccine and antibiotic preparations.

Traub (1947), Dinter (1948-1949), and Manninger (1951), working more or less independently and with different objects in mind, evolved a vaccine which offered a good immunizing potential and was safe in that it was a killed preparation. Preliminary investigations revealed that only a small number of strains of E. rhusiopathiae were capable of inducing immunity in host animals, but that these strains induced a good immunity against all strains of E. rhusiopathiae. Dedie (1949) was able to classify the strains of this organism into groups on the basis of their acid soluble antigens and was able to distinguish three such groups which he termed A, B, and N. He also noted that group B strains, which were the good immunizers, composed about 10 per cent of all the strains, and that groups A and N which, though they contained very virulent strains, induced little or no immunity. Dinter and Bakos (1947, 1948, 1949) observed that about 10 per cent of all strains possessed the capacity to agglutinate chicken erythrocytes. It thus appeared that the group B antigen, the immunizing antigen, and the haemagglutinating antigen were all present in the good immunizing strains, though separate from each other.

Traub (1947) selected good immunizing strains by exposing animals to selected strains and challenging them by the method developed

by Fortner (1944), which he cultured in liquid medium and killed with formalin. He then added aluminum hydroxide to the medium with the object of adsorbing the "specific immunizing substances" (S.I.S.) which were elaborated into the medium. This aluminum hydroxide adsorbed, formalin killed vaccine, produced from strains selected by Fortner's method of scarification, has several advantages. It is a killed preparation, good immunizing strains are present, it is concentrated, it is less expensive than the simultaneous serum culture method, and is, because of its nature, slowly adsorbed by the tissues, thus providing a longer period of antigenic stimulation. Numerous favorable reports have followed its application. Maas (1948) reported that the aluminum hydroxide formalin killed vaccine was 64 times more effective than the Muromcev vaccine. Possibly this can be explained in that the Muromcev vaccine consisted of a pool of many strains thus including poor immunizers with the good, and at the same time, diluting out any soluble antigens in the medium to the point of little value. Fortner (1947) was able to demonstrate good protection afforded by the Traub vaccine, using his own scarification procedure as a challenge method. Bergman (1949) also reported favorable results and demonstrated a greater potential than with the simultaneous method of Lorenz. Shuman (1952) of the United States Bureau of Animal Industries performed a number of experiments designed to test and evaluate the adsorbed vaccine and concluded that though his results could not be justly compared with those obtained in Europe, this vaccine was of definite value in protecting animals against swine erysipelas.

Manninger (1951) in Hungary developed a product very similar

to that produced by Traub (1947), but was working with an entirely different object in mind. Manninger was interested in knowing if the change from smooth to rough phase was accompanied by a permanent loss of virulence. Since E. rhusiopathiae shows quite a tendency toward dissociation, this organism was selected for study. He learned that Schoenig, Gochenour and Grey (1938) had immunized mice with rough strains and this motivated him to evaluate his own rough strains for their immunizing capacity. He found a few of his rough strains were suitable immunizers, and also that upon injecting as much as 50 ml of these organisms, there would be no subsequent signs of disease. Fortner's method of scarification was also found to yield no symptoms when these strains were used. Manninger felt that the vaccine products would be too rapidly eliminated from the tissues and that it would be desirable to adsorb these products onto some agent in order to prolong their action. He selected aluminum hydroxide. He observed that formalin did not appear to alter the antigenicity of his selected strains and, for the purpose of preserving the product, added this substance.

Thus he produced a vaccine very similar to that developed by Traub (1947) but by an entirely different approach. The one outstanding difference in the two products, and of the greatest advantage in the Manninger vaccine, is the use of rough avirulent strains, whereas Traub's preparations probably include virulent organisms. Even though formalin is added, some potential danger exists to a greater or lesser degree.

In testing his own vaccine, Manninger (1951) found that a good immunity developed after about 10 days in animals over 3-4 months

of age, this protection lasting for about 10 months.

Recent developments by Housemann (1949) and Gochenour (1952) were directed to the production of more stable vaccine products. Housemann produced a lyophilized adsorbate vaccine which is of value especially in the warmer climates, and Gochenour applied the lyophilization process to the standardization and preservation of cultures used in the simultaneous method of swine erysipelas prophylaxis and treatment. Gochenour (1952) reported that lyophilized vaccine dessicated from the frozen state retained its original viability and virulence for 2 years and remained stable in the restored state for 7 days.

Since the original recognition of E. rhusiopathiae as the etiological agent of swine erysipelas in Europe by Loeffler (1885) and by Creech (1921) in the United States, several effective procedures have been developed for the control of this disease. Though a number of shortcomings of these procedures continue to motivate present-day research efforts, perhaps the best summary of the available methods is to be found in the words of McNutt (1954): "The mistake we have made in the use of the simultaneous method is that we expect too much of it. If we will use it for what it is, we will get along much better because it is truely valuable and there is nothing better at present." Shuman (1954) observed: "This may also be true of the adsorbate bacterin." Shuman (1954) pointed out: "No prophylactic measure offers 100 per cent protection under all circumstances, and it is therefore the obligation of the practicing veterinarian of today to apply effectively those measures which are of present avail in the absence of more effective procedures which the future research efforts will doubtless reveal."

The application of ultrasonic wave action to bacterioclasis dates from the publication of Wood and Loomis (1927) of the destruction of luminous bacteria. Some of the main contributions have been made by Yen and Liu (1934), Stumpf, Green and Smith (1946) and Shropshire (1947). Liu and Yen (1934) and Royert and Grabar (1947) both observed the necessity for the presence of gas in a liquid as a precursor to bacterial destruction. The gas present did not need to be oxygen, as hydrogen and other inert gases served equally well, thus ruling out the possibility of an oxidation reaction as the disruptive agent. The actual mechanism responsible for the cellular disruption is believed to be that of cavitation. If the intensity of the sound waves is sufficiently high, the passage of these waves through a liquid results in the formation of minute cavities at regions of low pressure and their subsequent collapse in succeeding high pressure phases. As calculated by Rayleigh (1917), when cavitation occurs, pressures of several hundreds of atmospheres are developed around the collapsing cavities. This severe localized mechanical action is believed to be responsible for the disintegration of bacterial cells. Several investigators have reported on bacterioclasis e.g., Harvey and Loomis (1929), Chambers and Flosdorf (1936), Stumpf, Green and Smith (1946), Hamre (1949), and Williams and Gaines (1930).

The design of most modern equipment to dissipate the heat produced by the passage of the sound waves through the medium eliminates another factor which might be considered effective in cellular destruction and further delimits the possible active factors in sonic disintegration.

The influence of the suspending medium on the destructive effect

of sonic waves on bacterial cells has been evaluated in part by Beckwith and Weaver (1936), Jacobs and Thornley (1954) and Shropshire (1947). In general, they have observed some protection to be afforded the cells by such materials as milk, gum tragacanth and gelatin. Results also indicated that the power output served as an influencing factor in bacterioclastic action. Since power is a factor of the amplitude of the waves rather than the frequency, it was desirable in this work to use the upper limits of the sonic spectrum, as a greater amplitude is more easily obtained at a lower frequency. The action as far as the disruption of the cells may be concerned, is essentially the same. However, in using the upper limits of the sonic spectrum, one is able to take advantage of both a high frequency and a comparatively greater amplitude, or more generally speaking, a greater power output.

Strains of E. rhusiopathiae, SE-9 and SE-25 which were used in the production of two of the three experimental bacterins were obtained from the Corn States Laboratories located in Omaha, Nebraska. Strain 14774 which was used, both for the production of one experimental bacterin and for all of the experimental challenging procedures, was obtained from Dr. J.P. Newman of the Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan.

All three strains were tested for their fermentative ability, virulence for white mice, and haemagglutinative capacity.

The fermentative ability was determined by use of the basal medium described by Tiffany (1955) to which the various compounds to be tested were added. In general, all three strains displayed a good ability to ferment glucose, fructose, lactose, glactose and xylose, and a mediocre ability to attack arabinose and mannose.

The test for virulence was accomplished by injecting 0.1 ml of an 18 hr broth culture of the respective strains subcutaneously into each of five white mice. Each strain killed all five of the test animals within three days subsequent to injection.

The Hirst test was used to ascertain the ability of these strains to haemagglutinate chicken erythrocytes. In accordance with this test, the three strains were found to be negative with respect to inducing haemagglutination of the chicken red blood cells.

Pure cultures of these strains for use in this work were maintained at room temperature in semi-solid nutrient-tryptose broth. Tiffany and Wheaton (1954)

Preparation of Medium

Production of the vaccine necessitated the acquisition of large numbers of cells. It was also desirable to use a medium which was as free of other antigenic substances as possible and which would still support a good growth of the organism. In view of the successful use in our laboratory of nutrient-tryptose broth for the cultivation of E. rhusiopathiae (Tiffany and Wheaton 1954), and since it contained fewer potential antigens than some other acceptable media, it was selected for the cultivation of this organism for vaccine production.

Thirty-nine grams of dehydrated tryptose broth (Difco) and 12 grams of dehydrated nutrient broth (Difco) were added to 1500 ml of distilled water. These substances were dissolved with gentle heating. The dissolved medium was then filtered through four layers of gauze and a pledget of glass wool. After filtration, the medium was dispensed into three 3-liter flasks. At this point the pH was adjusted electrometrically to 7.6 with 0.5N NaOH, and a small (3.0 cm) glass enclosed bar magnet was placed in the flask. The flasks were gauze-plugged and the plug covered with a brown paper cap. Sterilization was accomplished by autoclaving at 121 C for 30 min. This medium was then cooled and ready for seeding.

Seeding of Culture Flasks

Seed cultures of each strain were prepared and checked for purity of culture by microscopic examination of stained samples of the respective cultures. After checking the purity of the seed cultures, 0.5 ml of this 18 hr growth was used to seed the respective flasks of medium prepared as described above.

The three 3-liter flasks, after seeding, were placed on separate

Mag Mix magnetic stirrers (Precision Scientific Company) at room temperature (approximately 24 C) and the rotations of the bar magnet were adjusted to about 260 r.p.m.

Incubation under these conditions was continued for 24 hrs. After 24 hrs, sufficient formalin (40 per cent) was added so as to produce a final concentration of 0.3 per cent. Incubation was then continued for 48 hrs.

A check for sterility was made following the 48 hr incubation period, during which the formalin was acting.

The cells were separated from the medium by centrifugation in an International Equipment Company Model PRI refrigerated centrifuge. This instrument was equipped with an angle head (R.C.F. 2787 X G). The medium fraction was placed aside prior to the addition of aluminum phosphate and the cells were resuspended in 50 ml of a solution of 1 per cent Bacto-peptone (Difco) and 0.3 per cent formalin. Using this formalinized peptone as a blank, a Cenco photelometer Model B-2 (using a flue filter) was adjusted to 100 per cent transmission. The density of the cell suspension was then adjusted to correspond with the No. 7 tube of the McFarland mephelometer (approximately 2.1 X 10^9 organisms/ml). The cells were then ready for sonic disruption.

Disruption of Cells

Bacterioclasis was accomplished with the use of a Raytheon Magnetostriction sonic oscillator Model DF-101. This equipment is designed to deliver 200 watts of power from the driver unit to the stand. The working frequency is 10 KC. The temperature in the cup (which contains the sample) is maintained at about 40-45 C by the use of water coolant flowing through a water jacket enclosing the cup. Approximately 1 qt/min is passed through this water jacket.

The unit was allowed to warm up for a period of 15 min, and a 50-ml sample (approximated) was placed into the cup. The machine was activated and adjusted to operating frequency. The sample was exposed to the sonic waves for 30 min. At the end of this time, the sample was removed and allowed to settle for 24 hrs at 36 F. An even, slightly opalescent suspension, colloidal in nature, resulted.

Subsequent to settling, the liquid suspension was removed and the debris discarded.

Aluminum phosphate was then added to both the medium, which was previously set aside, and the sonisized suspension.

Preparation of the Aluminum Phosphate

The aluminum phosphate was prepared by the addition of a solution of sodium phosphate to a solution of aluminum chloride.

Thirty grams of aluminum chloride was dissolved in 7,000 ml of distilled water. This solution was stirred continuously and 2,000 ml of 12 per cent sodium phosphate (hexa-hydrate) was added. Sufficient distilled water was added to make 15,000 ml. The flask was then covered and allowed to stand until the precipitate settled and then the supranatant was removed. Four 1-gal funnels equipped with No. 50 Watman filter paper were used to retain the precipitate while it was being washed with 8/10 liters of distilled water to remove the chlorides. The pH was adjusted to 6.0-6.5 using 1 N NaOH and the preparation allowed to stand for 2 hrs, at which time the pH was again checked. Sterilization of the precipitate was accomplished by autoclaving at 121 C for 30 min. During sterilization, the pH dropped to 5.4. The sterilized preparation containing 5 mg of aluminum (as aluminum) per ml was stored at room temperature prior to use.

The large quantity of precipitate was prepared in order to have sufficient amounts as to meet the needs of other projects.

Sufficient amounts of the precipitate were added to both the medium and the sonisized cells, so as to make a concentration of 3 mg/ml. The pH was checked to be sure that it was 5.2-5.4 and was so adjusted with 1 N NaOH if it was not found to be in this range. These preparations were then incubated at 35 C for 24 hrs.

The precipitate was removed from the medium by centrifugation and was recombined with the adsorbed sonisate, which had also been concentrated by centrifugation. These products were then suspended in a total volume of 10 ml of formalinized peptone solution (0.3 per cent formalin and 2.0 per cent Bacto-peptone). A check was made for sterility of the vaccine and if sterile, it was considered ready for evaluation.

Test Animals

White, male and female mice, between 16-20 grams, were selected for evaluation of the vaccines. These animals were provided with similar diets of animal pellets and water throughout the experimental work. They were observed daily, prior to, and during this work, and those animals which did not appear to be healthy were replaced or recorded as ill, if the signs of illness occurred within a day or so immediately following the inoculation of the vaccine.

Determination of the LD_{50}

Concurrently with the vaccine evaluations, the LD_{50} of the strain selected for challenge was determined. It was noted that strains of *E. rhusiopathiae* when suspended in 2 per cent Bacto-peptone (Difco), exhibited a greater virulence for mice than a similar suspension in

0.85 per cent saline solution. For this reason, greater attention was devoted to the evaluation of the LD_{50} for strains suspended in the peptone solution, as the challenge of an immune response was observed to be more severe than when 0.85 per cent saline solution was used as the suspending medium. A number of determinations of the LD_{50} for suspensions of E. rhusiopathiae in 2 per cent peptone were made. The result of each trial agreed well with the trials made concurrently with the vaccine evaluation tests, and the LD_{50} thus established was accepted for the challenge procedure.

Ten-fold dilutions of an 18-hr broth culture of E. rhusiopathiae were made in a 2 per cent peptone solution, ranging from 1-10 to 1-100 million. Five mice were selected to receive 0.2 ml of each dilution, respectively, by the subcutaneous route, and five mice receiving 0.2 ml of the peptone solution were held as controls. Deaths were recorded per each dilution for a period of 7 days, at which time observations were completed. The LD_{50} was then determined according to the method of Reed and Muench (1938). The LD_{50} as determined from the above procedure was found to be at a dilution of $10^{-5.6}$. It was necessary to use a clean pipette for making each dilution in order to avoid carrying organisms over to the next higher dilution which materially affected the resulting LD_{50}.

Inoculation of Vaccine into the Test Animals

In order to evaluate the potential of the proposed vaccine, a single 0.2 ml dose of the undiluted preparation and 0.2 ml of each of serial two-fold dilutions ranging from 1-2 to 1-256 were administered to the mice subcutaneously. Five mice were used per dilution. Formalinized peptone solution was again used as the diluting menstruum.

Two-tenths of a ml of the formalinized peptone solution was injected subcutaneously into five mice which were observed as controls along with five unvaccinated mice. The test animals were observed daily for deaths or ill effects of the vaccine. Two parallel groups of mice were thus injected.

Challenge of the Vaccinated Animals

Fifteen days after the administration of the vaccine, the two groups of test animals, and the controls, were challenged. One group received 0.1 ml of undiluted 18-hr broth culture and the other group 300LD_{50} contained in a volume of 0.1 ml. All injections were made by the subcutaneous route. Observations and records were completed at the end of seven days.

Evaluation of a Commercial Adsorbate Bacterin

In order to facilitate the comparison, the commercial adsorbate bacterin was tested by means of a procedure which was identical with the one described above for the mouse protection tests. The test animals were challenged in the same manner as were the animals used in the evaluation of the sonisate experimental bacterin, and the results were completed at the end of seven days.

Results and Discussion

The evaluation of any research concerned with the operation of biological systems is dependent upon a number of variables such as age, sex, weight, growth phase, etc. These variables can be more or less controlled by the investigator. However, biological systems are also invested with a number of unknown variables which influence the final results, and which, because of their unknown nature, do not lend themselves to regulation. Since this problem is concerned with a general evaluation, the role which the unknown variables play is much greater than if a more limited phase of the problem was evaluated. As an example of an unknown variable, one might consider the variation in response to antigenic stimulation of five mice. These animals may in all respects appear to be similar in age, sex, weight, etc., and yet give a different response to a standardized dose of an antigen. One must therefore keep in mind that though an attempt has been made to regulate the known variables, there are still other unknown variables which are active in affecting the final results, and which are not apparent in the tabulated experimental results.

It is interesting that the results of the mouse protection tests recorded in Tables I and II for bacterin 14774 showed no protection against the challenge doses employed. Yet the strain from which bacterin 14774 was made displayed a virulence for white mice which made it most acceptable as a challenging agent. This strain also had the strong capacity to attack certain of the carbohydrates which indicates the presence and operation of a number of enzyme systems. Strains SE-9 and SE-25, also virulent for white mice, were strong in their fermentative ability and provided a marked degree of protection against a challenge

Table I Results of challenging mice with 0.1 ml of a virulent undiluted culture of Erysipelothrix rhusiopathiae 15 days subsequent to inoculation with 0.2 ml of the respective bacterin dilutions.

No. of Mice	Dilution of Bacterin	Postvaccinal Deaths	Deaths from Challenge	Per Cent Survival
		Bacterin SE-9		
5	Undiluted	0	0/5	100
5	1/2	0	1/5	80
5	1/4	1	3/4	25
5	1/8	0	5/5	0
5	1/16	0	5/5	0
5	Unvaccinated Controls	0	5/5	0
5	Formalinized Peptone Controls	0	5/5	0
		Bacterin SE-25		
5	Undiluted	0	0/5	100
5	1/2	0	2/5	60
5	1/4	1	4/4	0
5	1/8	0	5/5	0
5	1/16	1	4/4	0
5	Unvaccinated Controls	0	5/5	0
5	Formalinized Peptone Controls	0	5/5	0

Results of mouse protection tests using bacterins SE-9 and SE-25.

Table I (Continued)

No. of Mice	Dilution of Bacterin	Postvaccinal Deaths	Deaths from Challenge	Per Cent Survival
		Bacterin 14774		
5	Undiluted	0	5/5	0
5	1/2	0	5/5	0
5	1/4	0	5/5	0
5	1/8	0	5/5	0
5	1/16	0	5/5	0
5	Unvaccinated Controls	0	5/5	0
5	Formalinized Peptone Controls	0	5/5	0
		Commercial Adsorbate Bacterin		
5	Undiluted	0	0/5	100
5	1/2	0	0/5	100
5	1/4	0	1/5	80
5	1/8	0	4/5	20
5	1/16	0	5/5	0
5	1/32	0	5/5	0
5	Unvaccinated Controls	0	5/5	0
5	Formalinized Peptone Controls	0	5/5	0

Results of mouse protection tests using bacterins 14774 and the commercial adsorbate bacterin.

Table II Results of challenging mice with 0.1 ml of a 1-1332 ($300LD_{50}$) dilution of a virulent culture of Erysipelothrix rhusiopathiae 15 days subsequent to inoculation with 0.2 ml of the respective bacterin dilutions.

No. of Mice	Dilution of Bacterin	Postvaccinal Deaths	Deaths from Challenge	Per Cent Survival
		Bacterin SE-9		
5	Undiluted	0	0/5	100
5	1/2	0	0/5	100
5	1/4	0	0/5	100
5	1/8	0	3/5	40
5	1/16	0	5/5	0
5	Unvaccinated Controls	0	5/5	0
5	Formalinized Peptone Controls	0	5/5	0
		Bacterin SE-25		
5	Undiluted	0	0/5	100
5	1/2	0	0/5	100
5	1/4	0	3/5	40
5	1/8	0	4/5	20
5	1/16	0	5/5	0
5	Unvaccinated Controls	0	5/5	0
5	Formalinized Peptone Controls	0	5/5	0

Results of mouse protection tests using bacterins SE-9 and SE-25.

Table II (Continued)

No. of Mice	Dilution of Bacterin	Postvaccinal Deaths	Deaths from Challenge	Per Cent Survival
		Bacterin 14774		
5	Undiluted	0	5/5	0
5	1/2	0	5/5	0
5	1/4	0	5/5	0
5	1/8	0	5/5	0
5	1/16	0	5/5	0
5	Unvaccinated Controls	0	5/5	0
5	Formalinized Peptone Controls	0	5/5	0
		Commercial Adsorbate Bacterin		
5	Undiluted	0	0/5	100
5	1/2	0	0/5	100
5	1/4	0	0/5	100
5	1/8	0	0/5	100
5	1/16	0	4/5	20
5	1/32	0	5/5	0
5	Unvaccinated Controls	0	5/5	0
5	Formalinized Peptone Controls	0	5/5	0

Results of mouse protection tests using bacterins 14774 and the commercial adsorbate bacterin.

dose of the organism.

Roots (1953) established that in the case of E. rhusiopathiae, virulence is no criterion for antigenicity. Dedie (1949) was able to classify strains of E. rhusiopathiae into three groups on the basis of their acid soluble antigens. He used the precipitin test, and designated these groups as A, B and N. He further observed that approximately 10 per cent of all strains which were tested fell into group B and this group included those strains which were antigenic. He further determined that the antibodies produced in response to these strains were also specific for strains which fell in groups A and N. Though groups A and N contained many very virulent strains, they were, for the most part, poorly antigenic. Roots (1953) observed that, under proper conditions, strains which could be classified into group A would elicit some degree of protection. He reasoned that since these organisms are markedly virulent and probably contain some traces of the B antigen, the increase in numbers of these strain organisms within a host would permit an increased amount of B antigen to stimulate the host and thus some protection was induced. Roots (1953) further reported that the immune response to group B strains could only proceed up to a certain level and that a higher level of protection could not be obtained under the conditions of his experiments.

In view of these previous investigations, the results listed in Tables I and II would seem to conform with these explanations. Strain 14774, showing no protection, was injected in the killed state, thus eliminating its virulence and permitting only the slight traces of B antigen to stimulate the host. This did not induce sufficient protection against the challenge doses. This strain, therefore, might

be considered to fall in either group A or N. Strains SE-9 and SE-25 induced a fair degree of protection, and having had their virulence eliminated also, show a predilection for the group B classification.

Dinter and Bakos (1948, 1949, 1951) in attempting to design some means of selecting antigenic strains of E. rhusiopathiae, observed that certain strains of this organism would agglutinate fowl erythrocytes. He observed that approximately 10 per cent of all strains of this organism possessed this property. He was later informed by Dedie that the organisms of the group B classification also had this property of haemagglutination.

It is interesting that the strains employed in this work all proved to be haemagglutination negative with chicken red blood cells. On the basis of antigenicity, the work reported herein tends to support the observations of Roots and Dedie, but in regard to the haemagglutination phenomenon, a directly opposite result was obtained.

The details of the haemagglutination test used by the European investigators were not available to the author and thus, in testing strains SE-9, SE-25 and 14774, it was necessary to use the Hirst test employed by virologists for diagnostic purposes. It is improbable that the original test involved a modification which would have affected the results to so great an extent and it is felt by the author that a more acceptable reason for the negative results might be obtained from a consideration of the medium in which the organisms were grown. It is an accepted fact that the addition of serum to a medium designed for the cultivation of E. rhusiopathiae tends not only to enhance the growth of the organisms, but also accents their fermentative ability. Since serum contains some enzymes which are able to attack carbohydrates

of polysaccharide nature and reduce them to the less complex molecules of di and monosaccharides, it is not a desirable component of fermentative media (Seaman and Woodbine 1952). However, a true evaluation of the effect of serum on the presence or absence of the haemagglutinating antigen has not been made. In all probability, the addition of serum to the medium used for the cultivation of the strains tested in this work might have made a difference on the haemagglutinative result. This is merely a supposition which occurs in attempting to explain the difference in results of this work and that of previous investigators relative to this haemagglutination phenomenon. This reasoning is felt valid since the addition of serum to a medium for cultivation of E. rhusiopathiae is a commoner practice in Europe than in the United States, and is the only logical basis apparent at present.

A closer inspection of Tables I and II reveals a slight variation in the protection conferred by bacterins SE-9 and SE-25. Against a challenge dose of undiluted culture, SE-9 afforded 100 per cent protection in the undiluted state only and 80 per cent protection in a 1-2 dilution of this bacterin. SE-25 was observed to have protected 100 per cent of the test animals against the undiluted culture challenge and only 60 per cent of the animals were protected in a challenge of the 1-2 dilution of this bacterin. This variation is more clearly exemplified in the response of the vaccinated test animals to a challenge dose of 300LD50, the results of which are presented in Table II. Here, bacterin SE-9 is observed to protect 100 per cent of the test animals in a dilution of 1-4, whereas SE-25 afforded 100 per cent protection in a dilution of 1-2.

Since both bacterins were prepared simultaneously and in a similar manner, one might be led to disregard the procedure as a source of variability and ascribe the variation to a difference in antigenicity of the strains. However, one must proceed with caution to such a conclusion, because of complexities of antigenicity and technique. Procedure, as a cause of variability, cannot be ruled out until more detailed information is available regarding the effect of such physical factors as adsorption, sonic disintegration and preservation. Nor can one completely absolve the factor of antigenicity without a more refined investigation of the two strains involved.

The magnitude of variation, therefore, might be attributed more safely to a combination of these factors, rather than to one alone.

One other aspect of this thesis problem which must be discussed is the reason for diluting the bacterin preparations and holding the challenge doses fixed. It would appear that one might hold the bacterin dose constant and vary the challenge doses, thus giving a more complete picture of the protection afforded by the experimental products. However, in Table I, it may be observed that the undiluted bacterins protected 100 per cent of the test animals against an undiluted culture challenge. For this reason, the procedure described in this paragraph was not feasible.

To this point, the discussion has been mainly concerned with an evaluation of the sonically disrupted, formalin killed experimental bacterins. Including now the results of the mouse protection tests obtained from the evaluation of the commercial bacterin, Tables I and II show that a greater protection was conferred by this product than by either of the two experimental bacterins.

The criterion for comparison being the degree of protection elicited by the bacterins, it is obvious that the commercial bacterin is the most acceptable of those tested. This is especially apparent in the response of the test animals to a challenge of 300 LD_{50}. The commercial product protected 100 per cent of the animals in a dilution of 1-8. The protection which the experimental products offered, though not as great as that produced in response to the commercial bacterin, was of considerable magnitude. In attempting to determine a reason for the differences, one is confronted with a number of variables. These variables which were evaluated only to an extent which would permit a standardization of procedure, and which would require much more time for a more complete evaluation, most probably effected a considerable portion of the differences in results obtained. For example, the process of sonic disruption of the cells introduces numerous variables which have not been elucidated sufficiently for the establishment of a set procedure. The length of exposure time, the nature of the suspending medium, the effect of this process on the antigenic components of the cells, and the nature of the resulting products as per their adsorption status, are all to be considered as a function of the sonic process. The object in using sonic disintegration in this work was based on an attempt to expose and make the antigenic components of the bacterial cell more readily available for stimulation of the host tissue. What actually happened, whether they were denatured or destroyed, is not known since the medium used for culturing these organisms was also adsorbed and recombined with the sonisized cells. In addition to the effect of the sonic disruption, one must consider the time element. Perhaps a longer time period for antigenic stimulation

might have caused better protection. For instance, one might have selected twenty days during which the bacterins had been permitted to act, thus inducing greater immunity. This would suggest a serological investigation of the titer variation. Perhaps two injections of the bacterins spaced at some predetermined interval might have produced a more desirable protection. Also, the selection of another more antigenic strain of E. rhusiopathiae might have been an influencing factor. These variables which, if properly evaluated, would perhaps result in the production of a more acceptable bacterin and would possibly narrow the gap between the results of the experimental and commercial products. This comparison is made with respect for the fact that a more acceptable commercial product may be produced.

The greatest over-all value of this work is the promise offered by the designed procedure as a means of producing an acceptable bacterin, and though much more work is indicated, such a promise in the field of erysipelas prophylaxis is indeed rewarding.

Summary

Three strains of Erysipelothrix rhusiopathiae were selected for the production of bacterins. The cells were killed by the addition of 0.3 per cent formalin to the cultures and were separated from the medium by centrifugation. These cells were then disrupted by sonic energy and adsorbed onto aluminum phosphate. The medium used for cultivation of the cells was also adsorbed with aluminum phosphate and the adsorbate products combined with the cell adsorbate. White mice were used to test these products against a challenge of undiluted virulent culture and against a challenge of 300LD_{50}.

Strain 14774 did not protect any of the test animals and strains SE-9 and SE-25 protected 100 per cent of the animals, injected with the undiluted bacterin, against the challenge of undiluted culture. Against a challenge of 300LD_{50}, bacterin SE-9 protected 100 per cent of the animals in a dilution of 1-4 and SE-25 protected 100 per cent of the animals in a dilution of 1-2 against a similar challenge.

A commercial bacterin was evaluated for the purpose of comparison and proved to offer greater protection than either of the experimental products. It protected 100 per cent of the animals in a dilution of 1-2 against a challenge with undiluted culture and 100 per cent of the animals were protected against a challenge of 300LD_{50} in a dilution of 1-8 of the commercial bacterin.

Considering the variables present, for which more refined evaluation is indicated, the most important development of this work was the promise which this procedure offers for the development of an acceptable bacterin.

Literature Cited

Beckwith, T.D. and Weaver, C.E. 1936 Sonic energy as a lethal agent for yeasts and bacteria. J. Bacteriol., 32, 361.

Breed, F. 1938 Swine erysipelas. Its distribution, increasing importance and control. J. Amer. Vet. Med. Assoc., 92, 344-355.

Chambers, L.A. and Flosdorf, E.W. 1936 Sonic extraction of labile bacterial constituents. Proc. Soc. Exptl. Biol. N.Y., 34, 631.

Cooper, M.S. 1954 Laboratory studies on the vaccination of mice and turkeys with an Erysipelothrix rhusiopathiae vaccine. Canad. J. Comp. Med., 18, No. 3, 83-92.

Creech, G.T. 1921 The bacillus of swine erysipelas isolated from urticarial lesions of swine in the United States. J. Amer. Vet. Med. Assoc., 12, 139-150.

Dedie, K. 1949 Saureloslichen antigene von Erysipelothrix rhusiopathiae. Monatsh. Vet. Med., 4, 7. Cited by M.S. Cooper, G.R. Personeus and B.R. Choman 1954 Laboratory studies on the vaccination of mice and turkeys with an Erysipelothrix rhusiopathiae vaccine. Canad. J. Comp. Med., 18, 83-92.

Dinter, Z. 1949 Haemagglutinationstest als eine Hilfsmethode bei der Bestimmung immunogener Rotlaufstamme. Berl. u. Much. tierarztl. Wochenschr, 177,. Cited by M.S. Cooper, G.R. Personeus and B.R. Choman 1954 Laboratory studies on the vaccination of mice and turkeys with an Erysipelothrix rhusiopathiae vaccine. Canad. J. Comp. Med., 18, 83-92.

Dinter, Z. 1948 Zur Schutzimpfung gagen Schweinerotlauf mit einer Adsorbatvakzine. Tierarztl. Umschau., 279, 17-18. Cited by M.S. Cooper, G. Personeus, Harvey, M.J. and Percival, R.C.

Laboratory studies on erysipelas. I, Immunization against swine erysipelas and susceptibility of swine to challenge. Amer. J. Vet. Res., 15, 594-598.

Dinter, Z. and Bakos, K. 1948 Deut. Tierarztl. Wchnschr., 55, 257. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Dinter, Z., Bakos, K. and Angermair, M. 1948 Berlin u. Munchen Tierarztl. Wchnschr., 32. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Dinter, Z. and Bakos, K. 1949 Ztschr. f. Hyg. u. Infektionskrank., 129, 263. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Emmerich and Mastbaum 1891 Cited from Friedberger, E. u. Pfeiffer, R. 1919 "Lehrbuch Der Mikrobiologie", 2, 900-901. Gustav Fischer, Publisher, Jena, Deutschland.

Fluckiger, G. 1949 Tierarztl. Umsch., 4, 89. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Fluckiger, G. 1952 Tierarztl. Umsch., 7, 84. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Fluckiger, G. 1952 Wien. Tierarztl. Monatsschr., 39, 197. Cited by

Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Fortner, J. and Dinter, Z. 1944 Ist das Rotlaufbakterium der alleinige Eggeger des Schweinerotlaufs? Z. Infektionskrank Haustiere., 60, 157.

Fortner, J. 1947 Tierarztl. Wchnschr., 61. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Fortner, J. 1947 Tierarztl. Wchnschr., 141. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review ofrecent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Fortner, J. 1947 Monatsh. f. Vet. Med., 2, 29. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Fortner, J. and Dinter, Z. 1944 Z. Ztschr. f. Infektionskrank., 60, 157. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Friedberger and Frohner 1910 Veterinary Pathology. Haye's authorized English translation. Hurst and Blackett, London, I, 221-243. Cited from Connell, R., Moynihan, I.W. and Frank, J.F. 1952 Studies of swine erysipelas. Part I: Literature review and survey of Erysipelothrix rhusiopathiae infection in Canada. Canad. J.

Comp. Med., 16, No. 3, 104-128.

Gledhill, A.W. 1949 Office of International Epizootics, 32, 5. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Gochenour, R.B. and Yotter, E.S. 1952 The stability of Erysipelothrix rhusiopathiae vaccine. (desiccated) Vet. Med., 47, 27-30.

Hamre, Dorothy 1949 The effect of ultrasonic waves upon Klebsiella pneumonae, Saccharomyces cerevisiae, Myagawanella felis and Influenza virus A. J. Bacteriol., 57, 279-295.

Hars, E. and Delpy, E. 1953 Inoculation de Erysipelothrix rhusiopathiae par voie intradermique. Application au titrage des vaccins et serums contra le rouget du porc. Bull. Vet. Acad. France 26, No. 5, 267-278.

Harvey, E.N. and Loomis, A.L. 1929 The destruction of luminous bacteria by high frequency sound waves. J. Bacteriol., 17, 373.

Hausmann, W. 1949 Deut. Tierarztl. Wchnschr., 56, 260. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, 380-388.

Hinshaw, W.R. 1955 Turkey disease research 1916-1955. Cited from "Teachers of Men", Michigan State University Press, 24-39.

Jacobs, S.E. and Thornley, Margaret J. 1954 The lethal action of ultrasonic waves on bacteria suspended in milk and other liquids. J. Applied. Bacteriol., 17, No. 1, 38-56.

Jastrzebskt, T. 1945 Muromcev's vaccine against swine erysipelas. Medycyna Weterynaryjna, 1, No. 5, 114-117. Cited by Kaplan, M.

1948 Production and use of new biological products against swine erysipelas in Poland. Vet. Med., 43, 174-179.

Kaplan, M. 1948 Production and use of new biological products against swine erysipelas in Poland. Vet. Med., 43, 174-179.

Koch, R. 1880 Investigations into the etiology of traumatic infective diseases. New Sydenham Soc. London, England. Cited from "Principles of Bacteriology and Immunity" Topley, W.W. and Wilson, G.S., Vol. I. 395, (1946). Williams and Wilkins Company.

Kondo, S. and Sugimura, K. 1935 The pathogenicity and immunizing property for swine of avirulent swine erysipelas bacillus obtained by treating with trypaflavin. J. Jap. Soc. Vet. Sci., 14, 338. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, No. 3, 380-388.

Leclainche 1897 Cited by Friedberger, E. u. Pfeiffer, R. 1919 "Lehrbuch Der Kikrobiologie" 2, 900-901. Gustav Fischer, Publisher, Jena, Deutschland.

Liu, S.C. and Yen, A.C.H. 1934 Further studies on the effect of ultrasonic waves on bacteria. Proc. Soc. Exptl. Biol. N.Y., 32, 485.

Loeffler, D. 1885 Experimentelle Untersuching uber Schweine Rotlauf. Ar. a.d. Kaiserl. Gesundhtshinte, I, 46. Cited by Connell, R., Moynihan, I.W. and Frand, J.F. 1952 Studies of swine erysipelas, Part I, Literature review and survey of Erysipelothrix rhusiopathiae infection in Canada. Canad. J. Comp. Med., 16, No. 3, 104-128.

Lorenz, Dr. 1893 Ein Schutzimpfungsverfahren gegen Schweinerotlauf. Cent. f. Bakteriol., 13, 357-364.

Lorenz, Dr. 1894 Schutzimpfungsversuche gegen Schweinerotlauf mit Anwendung eines aus Blutserum immunisierter Tiere hergestellten Impfpraparats. Cent. Bakteriol., 15, 278-285.

Lorenz, Dr. 1896 Die Bekampfung des Schweinerotlaufes durch Schutzimpfung. Cent. Bakteriol., 20, 792-796.

Maas, A. 1948 Monatsch. f. Vet. Med., 3, 61. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, 380-388.

Manninger, R. 1951 Acta Vet. Hung., 1, 5. Cited by Winter, H. 1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, 380-388.

Marsh, H. 1931 The bacillus of swine erysipelas associated with arthritis in lambs. J. Amer. Vet. Med. Assoc., 78, 57-63.

McNutt, S.H. 1954 Swine Erysipelas. Vet. Sci. News, 8, 1.

Muromcev and Matwijenko 1936 Sovj. Vet., 15, 20. Cited by Kaplan, M. 1948 Production and use of new biological products against swine erysipelas in Poland. Vet. Med., 43, 174-179.

Pasteur, L. and Dumas, M. 1882 Sur le rouget au mal rouge des porcs. C.R. Acad. Sci., 95, 1120-1121.

Pasteur, L. and Thuillier, L. 1883 La vaccination du rouget des porcs a l aide du virus morte attenue de cette maladie. C.R. Acad. Sci., 97, 1163-1171.

Ray, J.D. 1930 Arthritis in lambs and Erysipelothrix rhusiopathiae. J. Amer. Vet. Med. Assoc., 77, 107-108.

Ray, J.D. 1952 Transmissible diseases of swine. Vet. Med., 47, 9.

Rayleigh, L. 1917 On the pressure developed in a liquid during the

collapse of a spherical cavity. Phil. Mag., 34, 94. Cited by Jacobs, S.E. and Margaret J. Thornley. 1954 The lethal action of ultrasonic waves on bacteria suspended in milk and other liquids. J. Applied Bacteriol., 17, No. 1, 38-56.

Reed, L.J. and Muench, H. 1938 A simple method of estimating fifty percent end points. Amer. J. Hyg., 27, 493-497.

Roots, E. 1953 Die Grundlagen Der Rotlaufimmunitat. International Vet. Congress Proc., 1, 44-48.

Rouyer, M. and Grabar, P. 1947 Etude du mecanisme de l'action des ultrasons sur les microbes. Ann. Ins. Pasteur, 73, 215.

Schoening, H.W., Gochenour, W.S. and Grey, C.G. 1938 Studies on Erysipelothrix rhusiopathiae with special reference to smooth and rough type cultures. J. Amer. Vet. Med. Assoc., 92, 61-67.

Seaman, A. and Woodbine, M. 1952 Effect of sera on the fermentation reactions of Erysipelothrix rhusiopathiae. British Vet. J., 108, No. 3, 93-96.

Shropshire, R.F. 1947 Turbidimetric evaluation of bacterial disruption by sonic energy. J. Bacteriol., 53, 685.

Shuman, R.D. 1954 Experimental evaluation of swine erysipelas adsorbed bacterin. J. Amer. Vet. Med. Assoc., 124, No. 926, 362-367.

Shuman, R.D. 1954 Suggested application of present knowledge for the control of swine erysipelas. Proc. Book Amer. Vet. Med. Assoc., 91st annual meeting Aug. 23-26, 90-94.

Spencer, W.T. 1940 Sectional incidence of swine erysipelas in the United States. J. Amer. Vet. Med. Assoc., 99, 229-232.

Staub 1940 A new method of vaccination for the control of swine erysipelas. C.R. Acad. Vet. France, 13, 103. Cited by Winter, H.

1952 An improved swine erysipelas vaccine: a review of recent developments by European investigators. Cornell Vet., 42, 380-388.

Stumpf, P.K., Green, D.E. and Smith, F.W. 1946 Ultrasonic disintegration as a means of extracting bacterial enzymes. J. Bacteriol., 51, 487.

Tiffany, L.W. 1955 A suitable basal medium for evaluation of the fermentative capacity of Erysipelothrix rhusiopathiae. J. Vet. Res., 16, No. 1, 636-638.

Tiffany, L.W. and Wheaton, R. 1954 A versatile medium for cultivation of Erysipelothrix rhusiopathiae. J. Bacteriol., 68, 258.

Traub, E. 1947 Immunisierung gegen Schweinerotlauf mit konzentrierten Adsorbatimpfstoffen. Monatsh. Vet. Med., 2, No. 10, 165.

Veterinary Reporting Service, Michigan Department of Agriculture. 1, No. 12, December, 1954.

Voges, O. and Schutz, W. 1898 Z. Hyg. InfektKr., 28, 38. Cited by "Principles of Bacteriology and Immunity". Topley, W.W. and Wilson, G.S., Vol. I. 395, (1946). Williams and Wilkins Company.

Williams, O.B. and Gaines, N. 1930 The bactericidal effects of high frequency sound waves. J. Infectious Diseases, 47, 485-489.

Wood, R.W. and Loomis, A.L. 1927 The physical and biological effects of high frequency sound waves of great intensity. Phil. Mag., 4, 417. Cited by Jacobs, S.E. and Margaret J. Thornley. 1954 The lethal action of ultrasonic waves on bacteria suspended in milk and other liquids. J. Applied Bacteriol., 17, No. 1, 38-56.

Yen, A.C.H. and Liu, S.C. 1934 Effect of supersonic waves on bacteria. Proc. Soc. Exptl. Biol., N.Y., 31, 1250.

USE ONLY