STUDIES ON THE VIRULENCE OF LEPTOSPIRA POMONA

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STUDIES ON THE VIRULENCE OF LEPTOSPIRA POMONA

by

DIETRICH CHARLES BAUER

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I. INTRODUCTION

In the study of infectious diseases, a knowledge of the pathogenesis of the infection is of utmost importance. Observations on the pathogenicity and virulence of the etiological agent contribute to a better understanding of pathogenesis and the host-parasite relationship.

There is a lack of agreement among microbiologists on definite meanings for the terms "pathogenicity" and "virulence". Frequently the two terms are used as synonyms. This indicates the necessity for prefacing a study of these properties by assigning the definitions to be used throughout this thesis. A useful distinction between the two terms has been made recently by Miles (40). He suggests that "virulence" be used to refer to the observed infective capacity of the pathogen studied, when applied to the tissues of the host. The frequency with which virulent strains are found in a group of organisms determines whether the term pathogenic is applied to that group. "Pathogenicity" is therefore, no more than the disposition to virulence of a class of parasitic microbes.

For a definition of "virulence", Dubos (21) has presented the following: "Virulence is not a permanent intrinsic property of a given species. It expresses only the ability of a given strain of the infective agent, in a certain growth

phase, to produce a pathological state in a particular host, when introduced into that host under well defined conditions."

The importance of Leptospira pomona as a zoonotic agent has been well establisned (14, 17). The epidemiological, epizootiological and clinical aspects of this leptospirosis have been described in detail (66, 47, 52). However, information regarding the microbic factors which determine the virulence of this serotype is limited. A knowledge of these factors would aid in a more complete understanding of the patnogenesis of leptospirosis.

This study was undertaken to determine the virulence of two strains of Leptospira pomona for hamsters and guinea pigs, and to ascertain the changes in virulence of the organisms by passage through artificial fluid media. During the course of these studies, attempts were made to demonstrate in vivo adhesion of leptospirae to erythrocytes. Observations on the relationship between the in vitro production of a hemolysin and the virulence of Leptospira pomona were also made.

II. LITERATURE REVIEW

In 1936, Clayton, Derrick and Cilento (17) isolated a strain of leptospira from a farmer in Queensland, Australia which proved to differ serologically from known existing species. This species was designated "Leptospira pomona" by Derrick (20).

Morphology

Since morphological distinctions cannot be made between the various species of leptospirae (66), the description by Noguchi (49) of Leptospira icterohaemorrhagiae is applicable to L. pomona. He described the "spirochete" as being 7 to 40 microns in length with a diameter of about 0.25 microns. Spirals occurred at approximately 0.5 micron intervals. The terminal portions of the spirochete were described as pointed with one or both ends bent. Noguchi observed that reproduction took place by transverse division. He could not recognize flagella, axial filaments, or membranes. The organism was resistant to 10 percent saponin but dissolved readily in 10 percent bile salts.

More recent investigations with the electron microscope have elucidated previously undetectable structures. Morton and Anderson (48) were unable to observe any internal structure

in L. icterohaemorrhagiae. However, Babudieri (2) concluded from his studies that leptospirae consist of a cylinder of homogeneous protoplasm, enveloped by a very thin anistic membrane, wrapped around a rigid central filament, the axistyle.

In old cultures he found thin filaments, which he interpreted to be axistyles and residual masses which were freed by the destruction of the membrane and protoplasm. The axistyle was not affected by the papain, pepsin, or by freezing.

Bessemans and coworkers (6) investigated the granules which are found in old cultures, or in cultures subjected to temperature extremes, immune sera, or bile salts. By means of a pneumatic micromanipulator, single granules were isolated which could give rise to normal leptospirae. The resistance of these forms to noxious agents and heat was found to be no greater than that of leptospirae. Czekalowski (19) considers these granular forms to be phases in the life cycle of leptospirae. Other workers (2, 66) consider them to be merely degenerative products.

Webster and Reynolds (69) were unable to find any difference in the virulence for guinea pigs of long and short forms of L. pomona.

Cox and Larson (18) have recently reported on the colonial morphology of leptospirae. They described two types of leptospiral colonies. The first type is smaller in diameter

and more opaque than the second which is larger in diameter and translucent. Some strains were observed to produce colonies which developed irregular margins resembling the rough variant colonies of bacteria.

Virulence of Leptospirae

Many workers (66, 62, 23, 39, 15) have reported on the reduction in virulence of leptospirae due to cultivation in artificial media. Langworthy and Moore (39) transferred L. icterohaemorrnagiae at one to two week intervals in semi-solid medium. A perceptible decrease in virulence for guinea pigs was noted after two months, with a marked decrease in four to six months. Chang (15) found that L. icterohaemorrhagiae lost its virulence for guinea pigs after three to four transfers in semi-solid and fluid media over a period of 150 to 200 days. Faine (24) reported a 50 per cent lethal dose of 10 leptospirae for a culture of L. icterohaemorrhagiae which had been transferred 9 times in fluid media since its isolation from a guinea pig. Fifty percent lethal dose values of 4×10^5 cells for an eighteenth culture passage and greater than 10^9 cells were observed for a strain which had been maintained 2 1/2 years in culture. L. pomona was described by Webster and Reynolds (69) as losing its virulence for calves and guinea pigs after 3 to 4 months in semi-solid medium. Alexander et al. (1) reported that a strain of L. pomona lost its lethality for hamsters

after one year's passage in fluid medium. Rheinhard and Hadlow (52) found that L. pomona which had been maintained for three years in artificial medium, or which had undergone 100 serial passages in embryonating eggs was relatively avirulent for cattle.

Several procedures have been proposed for the maintenance of virulence of leptospirae in vitro. Stavitsky (61) found the virulence of L. icterohaemorrhagiae to be maintained for 100 days by freezing liver blocks from infected guinea pigs at -20 C. Freezing of cultures and 10 percent liver suspensions were unsuccessful in preserving virulence. Lyophilization of any of these materials did not maintain virulence. Chang (15) reported that the virulence of leptospirae could be maintained for 288 days when the organisms were grown in fresh liver enriched semi-solid medium. Bauer (24) observed that a culture of L. icterohaemorrhagiae was still virulent for guinea pigs after one year in semi-solid medium without transfer.

An increase in virulence of leptospirae may be attained by passage through susceptible animals. In 1917 Stokes, Ryle, and Tytler (64) reported that the virulence of L. icterohaemorrhagiae was increased by guinea pig passage. When guinea pigs were inoculated with blood from infected humans the average survival time of the animals was 10 days. After 14 passages through guinea pigs the average survival time was 4.8 days.

Noguchi (50) found that one passage of L. icterohaemorrhagiae through guinea pigs decreased survival time of the animals from 9-12 to 6-8 days.

Hamdy and Ferguson reported (30) that the virulence of L. pomona for hamsters was increased by serial passage. After sixteen passages, death occurred four days after inoculation. At the twentieth passage death occurred on the third day.

Van Thiel (66) reported that after eleven successive inoculations of L. pomona into guinea pigs, death occurred in 2 out of 22 animals.

Other workers have reported that attempts to increase virulence by animal passage were unsuccessful. Bernkopf et al. (5) passed a "bovine strain" of leptospirae through six passages in calves with little virulence increase. Borg-Petersen (9) could not detect an increase in the virulence of Leptospira bovis when passed through guinea pigs and mice.

Stavitsky (60) examined extracts from cultures of L. icterohaemorrhagiae for the presence of substances associated with virulence. He was unable to demonstrate hyaluronidase, fibrinolysin, leukocidin, or coagulase. However, Vollano and Brede (67) allowed filtrates from cultures of various pathogenic species of leptospirae to act on the synovial fluid of an ox. A decrease in the viscosity of this fluid, which is rich in hyaluronic acid, was noted. They interpreted their

findings as indicating the presence of hyaluronidase in the culture filtrates.

The presence of a leptospiral toxin had been suggested as early as 1917 when Inada (33) proposed that toxins arise from the disintegration of the spirochetes within the organs. Van Thiel (66) has described the clinical picture of leptospirosis as one of "general intoxication". Ferguson and coworkers (25) have proposed that the cause of fetal death and abortion in bovine leptospirosis is a hemolytic toxin.

Stavitsky (60) has described the findings of Japanese workers, in which toxic substances for guinea pigs were found in cultures of leptospirae kept under anaerobic conditions at 37 C. for 2 to 3 days. However, Stavitsky (60) was unable to demonstrate this toxin.

In 1956 Alexander et al. (1) observed the presence of a hemolysin in cultures of some species of pathogenic leptospirae. The hemolysin was described as being soluble, nondialysable, thermolabile and oxygen-stable. No antigenicity of the hemolysin could be demonstrated. Hemolytic activity was observed with sheep, cow, and goat erythrocytes.

This work was followed by the report of Russell (56) on the presence of a hemolytic agent in cultures of L. pomona. Characteristics similar to those previously described (1) were attributed to this hemolysin. The hemolysin was most active for sheep erythrocytes. Human and rabbit erythrocytes were

affected to some extent, while erythrocytes from guinea pigs showed little activity.

Chang (15) has suggested that the virulence of leptospirae is closely associated with the ability to attack fresh liver substances. He demonstrated that avirulent strains, isolated from guinea pigs, and grown in fresh liver enriched semi-solid medium regained some of their virulence for guinea pigs.

Stavitsky (62) has suggested that the surface potential of leptospirae may be associated with their virulence. However, there is no agreement on the sign of the surface charge of these spirochetes. Kligler and Aschner (36) found from electrophoretic studies that L. icterohaemorrhagiae was positively charged at the pH of blood. However, Brown and Broom (11), in a study of the adhesion of colloids to leptospirae in the presence of immune serum, found leptospirae to be negatively charged.

Stearns and Roepke (63) have demonstrated that dissociation forms of brucella have different electrophoretic mobilities.

The in vitro adhesion of leptospirae and other spirochetes to blood particulates and other bacteria has been observed. (11, 10, 12, 38). This adhesion takes place in the presence of immune serum and complement (68). Lamanna (37) has recently reviewed serological adhesion and discussed its biological significance.

Studies of genetic variation of leptospirae and its relation to virulence are limited. Schlossberger (57) reported in 1950, the demonstration of induced mutation of L. icterohaemorrhagiae. Using methods similar to those of Griffith (28) with pneumococci, Schlossberger observed that when a heat-killed virulent strain and an avirulent strain of L. icterohaemorrhagiae where incubated together for 3 days and then inoculated into a guinea pig, that the animal died and the virulent strain was isolated. However, attempts by this worker to demonstrate this phenomenon using other combinations of virulent and avirulent strains were unsuccessful.

Faine (24) found that one passage of a strain of L. icterohaemorrhagiae of reduced virulence through guinea pigs, restored the strain to its original virulence. He postulated that passage of leptospirae through susceptible animals results in the selective multiplication of virulent mutants.

Pathogenicity of L. pomona

Naturally occurring infections produced by L. pomona have been reported in man (14), cattle (3), swine (27, 8), horses (55), sheep (31, 58), and dogs (66).

The pig appears to be the principal or usual host of L. pomona (71). However, the clinical signs of the disease in swine are minimal, with the exception of abortion and death of newborn pigs (7).

The disease in cattle is characterized by abortions, decreased milk production, hemoglobinuria, anorexia and general depression (42, 43).

L. pomona infections in sheep result in fever, inappetance, and hemoglobinuria (45, 58).

Fever, temporary blindness, meningitis, and aching and swelling of the joints (66) are frequently reported for human L. pomona infections.

Hoag et al. (32) described experimental L. pomona infections in 2-day old chicks. Clinical symptoms were lacking. Leptospiremia of about 9 days duration was observed. Antibody production was weak in these animals.

Gleiser and coworkers (26) reported that the inoculation of 17-day old chicken embryos with L. pomona produced death in 2-5 days after hatching.

Ringen and Okazaki (53) reported that guinea pigs, hamsters, and white mice were readily susceptible; whereas young chicks were relatively resistant. The infection in hamsters is usually lethal (1); however, the virulence of L. pomona for guinea pigs is low (66, 70).

III. MATERIALS AND METHODS

The Effect of Medium Passage on the Virulence of L. pomona

Two strains of L. pomona were employed in this study. L. pomona (strain Wickard) was isolated (43) from the urine of an infected dairy cow and had been maintained in young guinea pigs for three years. This strain produced a febrile response in guinea pigs. It was not lethal for hamsters. Since its isolation, it had been used for experimental infections and was known to be pathogenic for cattle (47), swine (47), sheep (45), goats (47), and dogs (16).

L. pomona (strain Ohio) was isolated from an infected hog by workers at the Agricultural Experiment Station at Wooster, Ohio. It had been maintained in hamsters and guinea pigs and was lethal for hamsters. It had been used to produce experimental infections in sheep (45), hogs (46), and dogs (16).

The culture medium used throughout this study was a modified Chang's fluid medium (15) containing 0.01 per cent hemoglobin (Difco) and 10 per cent sterile rabbit serum. Each screw cap culture tube contained approximately 10 ml of media.

The leptospirae were transferred to fresh media when a culture had reached a stage of maximum growth. This determination was approximated by visual observation of the

turbidity of the culture and by periodic examinations by darkfield microscopy. Eight to twelve days were required for maximum growth to occur and approximately 5×10^8 cells per cc were observed. The inocula consisted of 0.1 to 0.5 ml of culture. All cultures were incubated at 29 C..

Virulence determinations were made during the logarithmic growth phase of a culture. The concentration of leptospirae in 5 to 10 day old cultures was ascertained by darkfield microscopy using a Petroff-Hauser bacterial counting chamber. The counting procedure consisted of placing a drop of culture on the counting cell with a capillary pipette and adjusting the cover glass over the drop. If the amount of fluid was insufficient to cover the cell area or was in excess resulting in the sample overflowing the cell, the cell was cleaned and the procedure repeated. When a satisfactory preparation had been obtained the chamber was placed on the stage of the darkfield microscope and allowed to remain undisturbed for several minutes to minimize any movement of the fluid. At a 420x optical magnification, and darkfield illumination, the number of motile leptospirae in 80, 1/400 square millimeter squares was determined. The 80 squares represented a group of 16 squares from each corner of the counting area and one group in the center. The number of leptospirae per square was calculated and multiplication of this figure by 2×10^7 gave the number of organisms per cc. The entire counting procedure was repeated at least once more to assure accuracy. The difference

between two or more determinations gave an error of 0 to 5 percent of the averages. When the concentration of a sample was too great for accurate counting, it was diluted so that from 2 to 6 leptospirae were seen per square. No increase in accuracy was obtained by decreasing the motility of the organisms with formalin. Therefore, only viable, motile leptospirae were counted. The error of this method for determining bacterial concentrations is reported to be about 10 percent (70).

When the number of leptospirae in the culture had been ascertained, ten fold serial dilutions were made. Dilution blanks containing 9.0 ml of Chang's medium minus the rabbit serum and hemoglobin were used. In some studies dilution blanks containing 13.5 ml or 13.0 ml of diluent were employed.

Five or seven Syrian hamsters were inoculated intraperitoneally with 1.0 cc of each dilution. In some titrations five guinea pigs were also inoculated intraperitoneally with 1.0 cc of each dilution. All hamsters and guinea pigs used were from animal colonies maintained in this laboratory and known to be free of leptospiroses. Hamsters were from 3 to 5 weeks of age, while the guinea pigs were 4 to 6 weeks old.

Hamsters were observed daily for signs of illness or death. When a hamster became moribund, 0.2-0.5 cc of blood was obtained by cardiac puncture. The sample was placed in a drop of sterile heparin sodium solution and centrifuged for 5 minutes at 2000 rpm in a Servall Small Type A centrifuge.

A sample of the plasma was examined by darkfield microscopy for the presence of leptospirae. In some cases the number of leptospirae in the plasma was determined using the counting chamber.

Twenty to thirty days following inoculation all surviving animals were sacrificed and a blood sample was obtained from each. A modified agglutination-lysis test (44) was conducted for each serum sample using living L. pomona antigen (strain Johnson).

Any animal which died three or more days after inoculation, or any which survived and had a serum antibody titer of 10^{-2} or greater was considered to have been infected.

The virulence of cells from each passage was expressed as that number of leptospirae which would kill 50 percent of the inoculated animals. This value will henceforth be referred to as the LD_{50}. The number of leptospirae infecting fifty percent of the inoculated animals will be termed the ID_{50}. LD_{50} and ID_{50} values were calculated by the method of Reed and Muench (51, 54).

A. Virulence Determinations with Strain Wickard

Virulence determinations with strain Wickard were conducted for medium passages 1, 4, 8, 10, 12, 16, 20, and 24. The first passage was a 32-day old culture of blood from an experimentally infected sheep. The sheep had been inoculated with blood from a guinea pig in the leptospiremic stage of the

disease. Contaminants were not observed in this culture when inoculations were made in Brain Heart Infusion Broth (Difco). The inoculum for the hamsters was centrifuged for 10 minutes at 2000 rpm in a Servall Small Type A centrifuge to remove gross particles prior to counting. Seven hamsters and five guinea pigs were used for each dilution.

To determine if the cell free culture fluid was antigenic and might interfere with the results, a portion of the culture was passed through a Seitz filter and then frozen and thawed twice in the refrigerator. Leptospirae were not visible after this treatment when viewed by darkfield microscopy. Ten fold dilutions of the filtrate were made and hamsters inoculated as previously described.

Five hamsters were inoculated with each dilution for passages 4 through 24. Passages 1, 4, and 8 were also inoculated into each of five guinea pigs. Virulence determinations for passage 16 were made using both washed and unwashed leptospirae. The organisms were separated from the culture fluid by centrifuging 40 ml of culture for 35 minutes at 12,000 rpm in a Servall Superspeed Centrifuge, Type SS-1. The sedimented leptospirae were then washed three times in 40 ml of sterile Chang's buffer (pH 7.0-7.2). The buffer consisted of 4.0 g $Na_2HPO_4.7H_2O$, 0.8 g KH_2PO_4, and 8.0 g NaCl in two liters of distilled water. The washed cells were resuspended in 40 ml of buffer and a virulence determination made. The supernatant fluid from the first centrifugation was passed through a Seitz

filter and frozen and thawed twice in the refrigerator. One cc of this preparation was injected intraperitoneally into each of three hamsters. Inoculation of the filtrate into Chang's medium produced no growth of leptospirae following 30 days incubation at 29 C.

Virulence determinations on media passages 4 and 8 were repeated. The leptospirae were isolated from another experimentally infected sheep.

B. Virulence Determinations with Strain Ohio

The first medium passage of strain Ohio was a 35 day old culture from infected guinea pig blood. Virulence determinations in hamsters were made on passages 1, 4, 7, and 11. Guinea pigs were used for passages 4 and 11.

C. The Effect of Age of Hamsters on Susceptibility to L. pomona

Two titrations were made using hamsters of various ages. The first study employed 7 week old and 4 week old hamsters. Each age group was inoculated with 10 fold dilutions of blood from guinea pigs in the febrile stage of leptospirosis. The second study employed 15 week and 4 week old hamsters. Blood from infected guinea pigs was again used for the inoculum.

D. Growth of Leptospirae *in vitro* after Passage in Media

The number of leptospirae needed to initiate growth in Chang's medium was determined for passages 1, 4 and 8 of strain Wickard. Counts and serial dilutions were made as previously described. The inocula consisted of 0.1 ml of fluid from each dilution blank and was transferred to each of five or seven tubes of medium per dilution. The cultures were examined by

darkfield microscopy for the presence of leptospirae at 10, 20, and 30 days. The number of leptospirae needed to initiate growth in 50 percent of the inoculated tubes was then determined.

Adhesion of Leptospirae to Erythrocytes

A. In vivo Adhesion

The premise that leptospirae might adhere to erythrocytes during leptospiremia was investigated using guinea pigs infected with the Wickard strain of L. pomona.

Infected guinea pigs which had temperatures of 104 F to 106 F were bled aseptically. Heparin was used as an anticoagulant. A portion of the blood was diluted ten fold, and each dilution inoculated into hamsters and Chang's medium as previously described. Five ml of the whole blood were centrifuged for 15 minutes at 2000 rpm in a Servall Small Type A centrifuge. The plasma was removed aseptically with a sterile capillary pipette. To it was added sterile Alsever's solution (59) to make a final volume of 5.0 ml. Ten fold dilutions of the plasma were made, and 5 hamsters and 5 tubes of Chang's medium inoculated with each dilution. The sedimented erythrocytes were washed three times by resuspending in 5.0 ml amounts of Alsever's solution and centrifugation for 15 minutes at 2000 rpm. After the final washing, the erythrocytes were resuspended to 5.0 ml volume in Alsever's solution. Ten fold dilutions were inoculated both into hamsters and media. Twenty to 25 days after inoculation the hamsters were sacrificed and a blood sample obtained. An agglutination lysis test was made on the sera. Antibody titers of 10^{-2} were considered

indicative of the presence of leptospirae in the inoculum. Cultures were examined after 10, 20 and 30 days of incubation.

As a control procedure, 0.3 ml of a culture of virulent L. pomona (strain Wickard) was added to 3.0 ml of heparinized blood from a non-infected guinea pig. The same separation, washing and dilution procedures were performed as described for the infected blood titration. Only Chang's medium was inoculated.

B. In vitro Adhesion

1. Two cc of blood were obtained by cardiac puncture from a guinea pig having a serum antibody titer of 10^{-5}. Blood was similarly obtained from a non-infected guinea pig. One ml of this was placed in heparin and centrifuged at 1000 rpm for 15 minutes. The plasma was removed from each sample with a capillary pipette. Guinea pig plasma, containing L. pomona antibody, was added to one portion of the normal cavian erythrocytes. The final volume equaled the original amount of blood or 1.0 ml. A similar amount of normal plasma was added to a second portion of normal erythrocytes. Both erythrocyte-plasma samples were incubated at 37 C. for 45 minutes. They were then centrifuged for 15 minutes at 1000 rpm and the plasma removed. The erythrocytes were washed twice with 2.0 ml amounts of Alsever's solution and resuspended to their original volume of 1.0 ml with fresh normal plasma. To each sample was added 0.1 ml of a culture of L. pomona

(strain Wickard). The culture contained approximately 1.6×10^8 organisms per cc. The final concentration was therefore approximately 1.5×10^7 leptospirae per cc. The samples were incubated for 15 minutes at 37 C. Following incubation the samples were centrifuged at 1000 rpm for 15 minutes and the leptospirae in the plasma counted using the Petroff-Hauser bacterial counting chamber. The erythrocytes were diluted approximately 1:10 and observed by darkfield microscopy for visible leptospirae-erythrocyte adhesion.

2. In a second *in vitro* study of adhesion, sheep erythrocytes and leptospiral antisera from sheep were used. The **L.** *pomona* antibody titer of the serum was 10^{-5}. The technique was as follows: 0.1 ml of culture of strain Wickard was added to each of six tubes. The six tubes contained respectively: 1) 0.9 ml of 0.85 percent saline, 2) 0.9 ml of whole blood, 3) 0.1 ml of erythrocytes in 0.8 ml of 0.85 percent saline, 4) 0.1 ml of erythrocytes in 0.3 ml of normal sheep sera which had been inactivated at a temperature of 55 C. for thirty minutes, 5) 0.1 ml of erythrocytes in 0.1 ml of *L.* pomona antiserum plus 0.7 ml of inactivated serum, and 6) 0.1 ml of *L. pomona* antiserum plus 0.7 ml of normal sheep sera. The tubes were incubated at 37 C. for 30 minutes followed by centrifugation for 15 minutes at 1000 rpm. The number of leptospirae were determined for the supernatant fluid from each tube using the counting chamber method. The erythrocytes

were diluted 1:10 and viewed by darkfield microscopy for leptospirae-erythrocyte adhesion.

3. The adhesion of L. pomona (strain Wickard) to polystyrene latex spheres (PSL) was investigated. The spheres were obtained from the Dow Chemical Company, Midland, Michigan. The diameter of the (PSL) was 1.171 microns. A distilled water suspension containing 7.2×10^8 spheres per cc was used in this study.

Chang's buffer, 0.1 ml, was placed in each of eleven tubes and 0.1 ml of strain Wickard culture was added to tubes 1, and 4-9. Tubes 1-9 contained 0.1 ml of PSL suspension. Tube 2 received 0.1 cc of normal sheep serum, while a similar amount of ovine L. pomona antiserum with an antibody titer of 10^{-5} was placed in tube 3. Tubes 4-6 contained ten fold dilutions of the normal serum. Tubes 7-9 had similar dilutions of antiserum. Tube 10 contained 0.1 ml of culture plus 0.1 ml of antiserum. Tube 11 had 0.1 ml of PSL and 0.1 ml of antiserum. The tubes were incubated for thirty minutes at 37 C. Following incubation 0.1 ml of PSL was added to tube 10 and 0.1 ml of culture to tube 11. A portion of each tube was then examined by darkfield microscopy for adhesion of leptospirae to the PSL.

In vitro Hemolysin Production in Relation to Virulence

Leptospirae were grown in the isotonic buffered medium described by Alexander *et al.* (1), augmented by the addition of 0.01 percent hemoglobin (Difco). This medium was inoculated with 0.5 ml of material from cultures of both strains of *L. pomona* which exhibited various degrees of virulence. The cultures were incubated at 29 C for 10 to 14 days. One to 3 days after growth had reached a maximum, the number of leptospirae was determined using the bacterial counting chamber. The cultures were then placed in the refrigerator (4 C.) until used.

Normal ovine and bovine blood were collected by venipuncture, haparinized and preserved in Alsever's solution at 4 C. Hamster, rabbit and guinea pig blood were obtained by cardiac puncture and similarly processed. The erythrocytes of the various species were prepared by washing three times with isotonic buffered base (pH 7.4). The base consisted of the isotonic buffered medium minus rabbit serum and hemoglobin. The washed red blood cells were then resuspended in sufficient isotonic base to make a 5 percent cell suspension by volume. A standard curve of percent hemolysis versus optical density was determined for the erythrocytes of each species. This was accomplished by adding 5.0 ml of distilled water to 5.0 ml of the above erythrocyte suspension. Centrifugation of this material at 2000 rpm did not cause sedimentation of the red

blood cells. Therefore, 100 percent hemolysis of the red blood cells had occurred. Appropriate amounts of hemolysed erythrocytes and isotonic base were mixed to give ranges of from 10 to 100 percent hemolysis. The final volume in each dilution was 1.0 ml. In order that sufficient amounts of sample were available for optical density determinations, 2.0 ml of isotonic base were added to each dilution, to make a total volume of 3.0 ml. Optical density determinations were then made in a Bausch and Lomb Spectronic 20 Colorimeter using matched test tubes. Readings were made at a wave length of 520 mu. Percent hemolysis was plotted versus optical density.

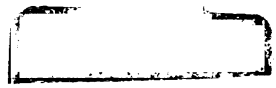
Ten L. pomona cultures of varying degrees of virulence were tested for hemolytic properties. The following is a list of the cultures used, their origin, and the number of transfers in media since isolation from an animal prior to their being tested for hemolytic properties.

1. Strain Wickard, from infected dairy cow, in medium passage for three years.
2. Strain Wickard, from infected guinea pig, third medium passage.
3. Strain Wickard, from experimentally infected sheep, third medium passage.
4. Strain Wickard, from experimentally infected sheep, fourth medium passage.
5. Strain Wickard, from experimentally infected sheep, ninth medium passage.

6. Strain Wickard, from experimentally infected sheep, tenth medium passage.
7. Strain Wickard, from experimentally infected sheep, twenty-fifth medium passage.
8. Strain Ohio, from infected guinea pig, fourth medium passage.
9. Strain Ohio, from infected guinea pig, sixth medium passage.
10. Strain Ohio, from infected guinea pig, eighth medium passage.

Since cultures 1 and 2 had not been included in previous virulence titrations 1.0 cc of each culture was inoculated intraperitoneally into each of three hamsters to determine if these cultures were infective.

Two fold serial dilutions of the cultures were made in the isotonic base. To 1.0 ml of each dilution, 1.0 ml of a 5 percent suspension of washed erythrocytes was added. As controls 1.0 ml of the washed erythrocytes were added to 1.0 ml of the diluent and of the medium. The tests were incubated at 37 C. for four hours and then placed in the refrigerator (4 C.) for twelve hours. Incubation has been reported to be necessary for complete hemolysis to occur (56). Following incubation, the tubes were gently agitated and centrifuged at 1000 rpm in an International Clinical Type Centrifuge model CL. One ml of the supernatant fluid was removed and placed in a colorimetric test tube. Two ml of isotonic base



was added and the optical density determined. Optical densities were plotted versus dilution. From the standard curve the optical density of a 50 percent hemolyzed erythrocyte suspension was determined. Using this value the dilution of culture necessary to produce 50 percent hemolysis of erythrocytes was ascertained. This dilution was used to express the hemolytic potential of a culture.

All ten cultures were tested using both sheep and hamster erythrocytes. Two fold culture dilutions of 1:2 through 1:10^{2}_{4} were used for the tests employing sheep erythrocytes, while culture dilutions of 1:2 through 1:128 were used when hamster erythrocytes were employed. Cow, rabbit and guinea pig erythrocytes were tested against cultures 1, 2, 7, and 8. Dilutions from 1:2 through 1:16 were employed.

IV. RESULTS

The Effect of Medium Passage on the Virulence of *L. pomona*

A. Virulence Determinations with Strain Wickard

The results of virulence determinations using strain Wickard are presented in Tables 1-12. Figure 1 depicts the changes in virulence which occurred following passage of the organisms in media. Unfortunately, titrations of the twelfth media passage in hamsters and the repeated titration of the eighth media passage did not include a dilution of leptospirae producing less than fifty percent infections. (Tables 5, and 3, respectively) For the calculation of LD_{50} and ID_{50} values of these titrations it was assumed that the next highest ten fold dilution of leptospirae would not have produced any infections. Therefore, these values may be higher than the actual value.

Examination of Tables 1-8 indicates that strain Wickard was not lethal for hamsters following one passage in media. A lethal effect was observed in titrations of the fourth through twenty-fourth media passages. However, hamsters exposed to the first passage were infected as demonstrated by development of serum antibody. Therefore, the virulence of the first passage was comparatively low. The relatively high

degree of infectivity of this passage is reflected by the ID_{50} value of 3.8 organisms (Table 15).

The virulence of the Wickard strain increased between the first and fourth passages. A decrease in virulence was observed for the eighth passage followed by an increase in virulence through the twelfth medium transfer. The sixteenth, twentieth and twenty-fourth passages indicated a steady reduction in virulence (Figure 1). When the fourth and eighth passages of strain Wickard, which had been isolated from another experimentally infected sheep (repeat titration), were titrated, an increase in virulence occurred after the fourth passage.

All hamsters and guinea pigs demonstrated antibody production when they were inoculated with a 10^{-2} dilution of twice frozen and thawed filtrate of the first culture passage. Fifty percent of the guinea pigs and hamsters inoculated with a 10^{-3} dilution of the filtrate experienced a serological response. Inoculation of higher dilutions did not produce antibody in either species. The leptospiral protein of the cell-free culture may have resulted in antibody production for the eighth passage titration in guinea pigs.

A comparison of the titrations of the sixteenth passage, in which a whole culture or washed cells were used as inocula (Table 6), indicates that the whole culture material possessed a lower LD_{50} value. Microscopic examination of the leptospirae after three washings in buffer revealed that many of the organisms had become granular, and some cellular

disintegration had occurred. In the counting of these leptospirae, attempts were made to include only those organisms which were intact and had retained their normal morphology and motility. Hamsters inoculated with filtered, frozen and thawed supernate of the culture material did not display any observable signs of infection. Sera from these animals did not contain demonstrable L. pomona antibody.

Hamsters and guinea pigs were equally susceptible to the eighth passage inoculum, whereas hamsters were slightly more susceptible to organisms of the first and fourth passages.

B. Virulence Determinations with Strain Ohio

Changes in virulence similar to those observed for strain Wickard were noted. Tables 13-17 represent the results of strain Ohio titrations. Figure 1 demonstrates the rapid decrease in virulence from the first to the fourth passage. The LD_{50} values of these two titrations were 1 and 15,000, respectively. The virulence of strain Ohio increased with the seventh and eleventh passages at a rate similar to that of strain Wickard. A 50 percent end point was not reached with the first passage. The LD_{50} value was determined as previously described.

Hamsters and guinea pigs were essentially equally susceptible to infection with this strain.

Figure 2 illustrates the relationship between the size of the inoculum and the duration of survival of infected

hamsters. The plotting of these data results in essentially a straight line. From the slope of this line the generation time of L. pomona in vivo may be calculated using the method of Youmans and Youmans (72). The generation time is equal to the log of 2 divided by the reciprocal of the slope. This method of calculating generation times in vivo is based on the assumption that when death occurs a constant number of organisms is present. Studies with L. icterohaemorrhagiae infection in guinea pigs have indicated that this relationship is present (24). In the present study leptospiral counts for plasma from moribund hamsters ranged from 3×10^6 to 10^3 organisms per cc. Since the number of leptospirae in the blood at death has been reported as being from 50 to 70 percent of the total number of organisms in the body (25), the range of organisms in the plasma probably represents a relatively constant leptospiral population in the hamster at the time of death.

A comparison of the dosage-response curves of leptospirae possessing comparatively high and low degrees of virulence, indicates that the slopes are relatively equal but that death of an animal inoculated with organisms of lower virulence was delayed about 50 hours. The fact that the curve of the less virulent culture is not linear near the LD_{50} inoculum is probably due to the interference of antibody. The calculated generation time of L. pomona in hamsters was found to be 7.5 hours.

The majority of hamsters died between 4 and 12 days following inoculation. Examination of plasma from hamsters which died within this period always revealed the presence of leptospirae. However, in a few cases hamsters died 12 to 18 days following inoculation. Leptospirae were not seen in the plasma of these animals, but antibody was present as determined by the agglutination lysis test.

C. The Effect of Age on Susceptibility of Hamsters to L. pomona

Table 16 records the results of two determinations on the susceptibility of 3 and 15 week old hamsters to L. pomona as compared with 4 week old animals. Eight week old hamsters were slightly more susceptible than the 4 week old animals. It was calculated that a $10^{-3.3}$ dilution of guinea pig blood would infect 50 percent of those 3 weeks old, whereas a $10^{-3.4}$ dilution would produce the same percentage of infections in the 4 week old hamsters. Fifteen and 4 week old hamsters were equally susceptible. A dilution of guinea pig blood of $10^{-3.6}$ was calculated to be the dilution which would produce 50 percent infection in each age group.

D. Growth of Leptospirae In Vitro after Passage in Media

Table 17 summarizes the results of this study. The number of organisms necessary to produce growth in 50 percent of the inoculated tubes is: 2.4 for the first passage, 20.5 for the fourth passage, and 22.9 for the eighth passage.

Adhesion of Leptospirae and Erythrocytes

A. In vivo Adhesion

The results of leptospirae-erythrocyte adhesion studies using strain Wickard are summarized in Table 16. Titration of the blood components in hamsters indicated that equal numbers of leptospirae were in the plasma and washed erythrocyte portions. When the titration was made in Chang's medium, a slightly higher concentration of leptospirae was noted in the erythrocyte portion than in the plasma. The control study, employing normal guinea pig blood to which the Wickard strain culture had been added, indicated that the organisms were equally distributed between the erythrocytes and plasma.

Microscopic examination of blood from sheep, guinea pigs and hamsters obtained during the leptospiremic phase of infection did not reveal adhesion of leptospirae to erythrocytes. Adhesion of leptospirae to achromacytes was observed in 7 to 30 day old Chang's medium cultures of infected blood from various animals. Investigation of adhesion by visual methods is often complicated by the presence of filamentous elements attached to, or projecting from, erythrocytes. The occurrence of these "pseudospirochetes" and methods for distinguishing them from true spirochetes have been reported (29).

B. In Vitro Adhesion

1. When the supernatant fluid was separated from the erythrocytes which had been incubated with normal plasma, it was found that it contained over 90 percent of the original number of leptospirae. Less than ten percent of the original number of organisms remained in the fluid removed from erythrocytes incubated with plasma which contained L. pomona antibody. An examination of diluted preparations of sedimented erythrocytes by darkfield microscopy, failed to reveal leptospirae in either the preparation of red blood cells incubated with normal or with immune plasma..

2. Table 22 summarizes the results of the second trial to demonstrate in vitro adhesion of erythrocytes and leptospirae. No adhesion was demonstrated. If adhesion had taken place, tube 6 containing antibody and complement would have contained fewer leptospirae than tube 5 to which no complement was supplied. The fewer numbers of organisms in these tubes containing L. pomona antiserum was probably due to lysis of the leptospirae by lytic antibody. Examination of resuspensions of the sedimented erythrocytes from each tube did not show erythrocyte leptospirae adhesion.

3. Adhesion of leptospirae and polystyrene latex spheres was not demonstrated in any of the tests.

In vitro Hemolysin Production in Relation to Virulence

The twenty-fifth media passage of the Wickard strain demonstrated the greatest hemolytic activity. The virulence of this culture was relatively low. The fourth passage of this strain produced approximately one-third the hemolysin of the twenty-fifth passage and yet was considerably more virulent. Culture 2 (Table 20) which was not lethal for hamsters, demonstrated more hemolytic activity than many of the more virulent (lethal) cultures. The culture of the Wickard strain which had been in media for three years produced the lowest concentration of hemolysin. This culture stimulated antibody formation in hamsters. The third passage of this strain which was isolated from a sheep, also produced low hemolysin concentrations. Cultures of the relatively virulent ninth and tenth passages of the Wickard strain (repeat titration) indicated only moderate hemolytic activity.

A definite relationship between virulence and hemolytic potency was also absent with strain Ohio. Cultures of the sixth and eighth passages of this strain exhibited approximately 50 percent of the hemolytic activity of the less virulent fourth passage. A correlation between the hemolytic potential and the age of the culture, number of organisms in the culture at the time of deactivation, or number of passages in media, was not evident. Table 20 summarizes the results of this study.

Table 21 indicates that L. pomona cultures were hemolytically active against cow, rabbit, and hamster erythrocytes. Guinea pig erythrocytes were not susceptible to hemolysis. Cow and sheep erythrocytes had relatively equal susceptibilities. Rabbit cells were slightly more sensitive than those from hamsters. Differences between the comparative hemolytic activity of the various cultures for the erythrocytes of the different animals and those of sheep were not observed.

V. DISCUSSION AND CONCLUSIONS

The Effect of Medium Passage on the Virulence of L. pomona

A and B.

The rapid decrease in virulence observed for both L. pomona strains following cultivation in media is in agreement with previous reports (66, 71). However, the fact that increases in virulence may occur following several medium passages of the leptospirae has been less frequently reported. Van Riel has been cited (66, 1) to have observed an increase in virulence of a strain of L. icterohaemorrhagiae for guinea pigs after 8 months cultivation in media. In the present study the increases occurred for the fourth and tenth passages of strain Wickard and for the seventh of the Ohio strain. The repeated titration using strain Wickard indicated that an increase in virulence had occurred for the eighth transfer.

Alterations in virulence are usually explained as a result of changes in the number of virulent organisms in the inoculum rather than as an alteration in the virulence of each organism (21, 24). Continuous guinea pig passage of strain Wickard may have lessened the original or inherent virulence for hamsters. The single passage of the organisms in sheep may have selected a few virulent leptospirae which when transferred

in media reproduced and were in sufficient numbers in the fourth passage to cause death of hamsters.

The increases in virulence observed with both strains after 4 to 8 medium passages may be due to a mutation. The culture medium would seem to favor the proliferation of the virulent mutant over the less virulent organisms. The subsequent decrease in virulence observed after 12 medium passages of strain Wickard indicates either a loss of the ability of the mutant to outgrow the parent cells or that another mutation to a less virulent population had occurred.

Hamsters which survived the inoculum usually failed to demonstrate serum antibodies. It is evident that the lethal leptospirae must have constituted the greatest percentage of individuals in these inocula. The only exception was observed for hamsters inoculated with the first medium passage.

Regardless of the mechanism involved in the virulence changes of these two strains, the change does not seem to be complete and is reversible.

Morse (41) has observed that swine inoculated with the nonlethal strain Wickard shed leptospirae in their urine which occasionally killed hamsters. Hamdy and Ferguson reported an attenuation of a strain of L. pomona for cattle after several hamster passages (30).

The fact that passage of strain Ohio in guinea pigs did not seem to effect its virulence for hamsters may be because this strain was in continuous guinea pig passage for

about 8 months prior to the initiation of virulence determinations, as compared to about 3 years for strain Wickard. Therefore, the length of time that the strain is maintained in guinea pigs may determine whether alterations in the virulence of the organism will take place. It is possible that strain Ohio and strain Wickward possess different virulence factors. Guinea pig passage may have an effect on just a certain factor or factors. Recent observations have shown a difference in the in vivo hemolytic activity of the two strains for hamsters (65). This may indicate a dissimilarity in one or several factors associated with the pathogenic properties of these strains.

When the LD_{50} values observed for washed and unwashed cells of the sixteenth passage were compared, it was difficult to assess the observed difference. This was due to the disruption of the organisms during the washing procedure. Although abnormal cells were not included in the enumeration, it is very likely that organisms incapable of reproducing were included among those counted. Due to these factors it does not seem justifiable to attribute a greater virulence to the unwashed leptospirae. A soluble agent capable of producing observable illness was not demonstrated in the cell free culture fluid of this passage.

A straight line was obtained when the average survival time of infected hamsters was compared to the number of organisms inoculated (Figure 2). From the slope of the line the

in vivo generation time of both strains was calculated to be 7.5 hours. The generation time of *L. icterohemorrhagiae* in guinea pigs has been reported as 8.3 hours (24). The relationship would appear to be significant.

The increase in the average survival time of hamsters inoculated with a less virulent culture is probably due to there being fewer virulent organisms in this inoculum. Since there seems to be a constant number of leptospirae in an animal at death, a greater period of time would be required for the fewer virulent organisms in this inoculum to multiply to the required population.

The observation that some hamsters die 1-6 days after the leptospiremic phase of the disease and have demonstrable antibody in their sera is in accord with the suggestion by Faine (24) that the lesions of leptospirosis may develop to such a degree that they cannot be repaired or compensation cannot occur.

Hamsters appeared to be slightly more susceptible than guinea pigs to infection with leptospirae of the early medium passages of the Wickard strain. The findings are in contrast to the report of Ringen and Okazaki (53), who found the guinea pig to be the more susceptible laboratory animal. The disagreement may be due to the fact that these workers used a strain of *L. pomona* which had been cultivated in media for several years. Other factors may be involved such as the strain of animals used or the strain of *L. pomona* employed.

C. The Effect of Age on the Susceptibility of Hamsters to L. pomona

Only slight differences in susceptibility to L. pomona (strain Wickard) were observed for 4, 8, and 15 week old hamsters (Table 16). When susceptibility is based on the period of survival of infected hamsters rather than the number of organisms needed to produce infection, 3-5 week old hamsters have been reported to be more susceptible than older animals (30). The age of hamsters which may be employed in the diagnosis of leptospirosis does not seem to be limited to weanlings if infection in these animals is determined by development of antibody.

D. Growth of Leptospirae In vitro after Passage in Media

The results (Table 17) indicate that the number of leptospirae needed to initiate growth in Chang's medium increased as the organisms were transferred in media. These findings are not consistent with the theory that as bacteria are transferred in media they become adapted to the media by the selection of adapted variants.

The results may be due to differences in the media used for each titration. The medium component that is least under experimental control is the rabbit serum. The increased number of organisms needed to promote growth after 4 and 8 medium passages may be associated with the fact that these

passages were lethal for hamsters in contrast to the first passage.

Adhesion of Leptospirae and Erythrocytes

A. In vivo Adhesion

L. pomona (strain Wickard) was equally distributed between the plasma and washed erythrocyte portions of infected guinea pig blood as determined by titrations in hamsters. Titrations in media indicated a slightly higher concentration of leptospirae in the erythrocyte portion. Equal concentrations of leptospirae were found in the erythrocyte and plasma portions of normal guinea pig blood to which the leptospirae had been added. Leptospirae were not observed adhering to erythrocytes during leptospiremia in guinea pigs, hamsters, and sheep.

It was not possible, therefore, to unequivocally demonstrate that adhesion was commonplace during leptospiremia. The presence of leptospirae in the erythrocyte portion was probably due to some extent to the speed of centrifugation. It was felt that 2,000 rpm for 15 minutes was necessary to insure complete separation of the erythrocytes and plasma. Other factors may be involved such as an electrostatic attraction of leptospirae and erythrocytes or adherence of the organisms to leuckocytes and platelets present in the centrifugate.

B. In vitro Adhesion

1. The results of this experiment indicated a removal of over 90 percent of the leptospirae by the antibody treated erythrocytes. However, leptospirae were not observed by darkfield microscopy when a systematic search was performed on diluted suspensions of the erythrocytes. Approximately 10^6 organisms per cc would have been present in the diluted suspensions, a number which is readily detectable microscopically. It would seem that a large number of leptospirae had been destroyed. Lytic antibody present on the erythrocytes or incompletely removed by washing seems to be the likely cause of the destruction of the leptospirae.

2. The results of this experiment (Table 19) also seem to be influences by lysing of the leptospirae by antibody. Complement is necessary for in vitro adhesion to take place (37) but does not seem to be required for the lytic action of leptospiral antibody (71). The approximately equal reduction in the number of organisms in all tubes of the series which contained antiserum indicates that complement is not necessary for this reduction to take place. In all cases the reduction is probably due to lysing of the leptospirae.

3. Polystyrene latex spheres did not seem to be a suitable material for the adhesion of leptospirae. Lamanna (37) reported that adhesion of bacteria and organic colloids has been observed.

In vitro Hemolysin Production in Relation to Virulence

The hemolytic potency of the cultures examined seemed to be independent of the virulence of the culture for hamsters. A culture of strain Wickard which was not lethal for hamsters produced a greater concentration of hemolysin than several other passages of the strain which were lethal. The culture which demonstrated the highest LD_{50} produced the greatest hemolysis.

Determinations of LD_{50} were not made for each of the cultures examined. The virulence of these cultures was assumed to be represented by the curve shown in Figure 1. Virulence changes may have occurred between the points on this graph. However, no more than 1 to 3 passages were made between virulence determinations and it does not seem likely that marked alterations occurred within these limits.

An explanation for the differences in hemolytic activity of the various cultures could not be ascertained from the experimental results. However, the suggestion has been made that mutations of the leptospirae occurred during the course of the investigation. The changes in hemolytic potency may be the result of differences in the metabolic activity of the mutants and parent cells.

The differences in susceptibility of erythrocytes from several animal species to the hemolytic factor of *L. pomona* is in agreement with previous reports (56). *In vivo* hemolytic

activity in hamsters did not appear to correlate with the *in vitro* hemolytic activity of the strain for hamster enthrocytes. The previously mentioned hematological examinations (65) of strain Ohio infected hamsters gave no indication of a hemolytic type of infection. However, the Ohio strain produced comparatively significant amounts of hemolysin *in vitro*.

The results of the present study indicate that the virulence of *L. pomona* may increase when the organisms are transferred in Chang's medium. Future work concerning the effect of various media on virulence and comparative growth rates of virulent and avirulent strains in media are indicated. There is a possibility that an alteration in the virulence of *L. pomona* may occur when the organisms are maintained in guinea pig passage. The inoculation of sheep with the nonlethal strain may favor the proliferation of a few lethal mutants. The effect on virulence of successive passages of a given strain in a single host species may be responsible for alterations in infectivity and lethality fo the "passage species" and other species. This would be important in the epizootiology of leptospirosis. The recently reported medium for observing the colonial morphology of leptospirae (18) might facilitate the investigation of leptospiral variation. Unfortunately, repeated attempts in this laboratory to cultivate leptospirae, known to be virulent, on this medium were unsuccessful.

It has been suggested that the hemolytic activity of the leptospirae is an attribute of a hemolytic toxin (1). The results of the present study did not unequivocally demonstrate a relationship between the hemolytic activity of L. pomona and its virulence for hamsters. If the hemolytic activity is due to a toxin, it would seem that this is only one of several factors concerned in virulence. In order to assess the role of the hemolytic factor in the course of leptospirosis, the purification and characterization of the hemolysin will be required. Subsequent demonstration that the purified toxin (hemolysin) contributes per se to the course of the infection might then be afforded.

VI. SUMMARY

An investigation was made on the effect of medium passage on the virulence of two strains of L. pomona for hamsters and guinea pigs. Strain Wickard which originally produced only a mild infection in hamsters, was lethal for these animals after 4 passages in media. A decrease in virulence was observed in the early medium passages of both strains, followed by an increase in virulence after 4 to 8 transfers. After 12 passages, strain Wickard demonstrated a steady decrease in virulence. Similar changes in infectivity for guinea pigs were noted. It was suggested that the changes in virulence may be the result of mutations of the leptospirae.

Hamsters were slightly more susceptible to L. pomona than guinea pigs. Four, 7 and 15 week old hamsters were essentially equally susceptible to infection.

The in vivo adhesion of leptospirae and erythrocytes was not unequivocally demonstrated. In vitro studies did not reveal adhesion, although the presence of lytic antibody may have influenced the results.

The virulence of the various medium passages was not related to the ability to produce hemolysin in vitro. The susceptibility of hamster erythrocytes to the hemolysin from

the various cultures did not seem to be associated with the ability of these organisms to produce a hemolytic effect in hamsters.

TABLES

Tables 1-14. *Average number of days after inoculation that animals survived.

**Numerator = number of animals infected; Denominator = number of animals inoculated.

TABLE 1

TITRATION OF STRAIN WICKARD IN HAMSTERS,

MEDIA PASSAGE NO. 1

Number of Leptospirae per Inoculum	Number of Animals Infected	Number of Deaths	Average Survival Time*
1.22×10^4	7/7**	0	-
1.22×10^3	7/7	0	-
1.22×10^2	7/7	0	-
1.22×10^1	6/7	0	-
1.22	1/7	0	-

TABLE 2

TITRATION OF STRAIN WICKARD IN HAMSTERS, MEDIA PASSAGE NO. 4

Number of Leptospirae per Inoculum		Number of Animals Infected		Number of Deaths		Average Survival Time*	
Original Titration	Repeat Titration	Original Titration	Repeat Titration	Original Titration	Repeat Titration	Original Titration	Repeat Titration
1.02×10^4	3.80×10^5	5/5**	5/5**	5	5	11.4	12.4
1.02×10^3	3.80×10^4	2/5	5/5	2	5	12.5	8.0
1.02×10^2	3.80×10^3	0/5	5/5	0	4	--	10.8
1.02×10^1	3.80×10^2	0/5	0/5	0	0	--	--
1.02	3.80×10^1	0/5	0/5	0	0	--	--

TABLE 3

TITRATION OF STRAIN WICKARD IN HAMSTERS, MEDIA PASSAGE NO. 8

Number of Leptospirae per Inoculum		Number of Animals Infected		Number of Deaths		Average Survival Time*	
Original Titration	Repeat Titration	Original Titration	Repeat Titration	Original Titration	Repeat Titration	Original Titration	Repeat Titration
7.46×10^6	3.82×10^6	5/5**	5/5**	5	5	7.6	6.8
7.46×10^5	3.82×10^5	5/5	5/5	5	5	9.0	10.6
7.46×10^4	3.82×10^4	0/5	5/5	0	4	--	10.8
7.46×10^3	3.82×10^3	0/5	5/5	0	4	--	13.8
7.46×10^2	3.82×10^2	0/5	3/5	0	2	--	12.0

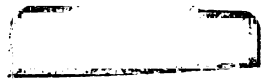


TABLE 4

TITRATION OF STRAIN WICKARD IN HAMSTERS, MEDIA PASSAGE NO. 10

Number of Leptospirae per Inoculum	Number of Animals Infected	Number of Deaths	Average Survival Time*
2.45×10^7	5/5**	5	4.8
2.45×10^6	5/5	5	5.8
2.45×10^5	5/5	4	7.0
2.45×10^4	3/5	3	9.6
2.45×10^3	0/5	0	--

TABLE 5

TITRATION OF STRAIN WICKARD IN HAMSTERS, MEDIA PASSAGE NO. 12

Number of Leptospirae per Inoculum	Number of Animals Infected	Number of Deaths	Average Survival Time*
2.4×10^7	5/5**	5	4.2
2.4×10^6	5/5	5	6.4
2.4×10^5	5/5	5	7.6
2.4×10^4	5/5	5	10.2
2.4×10^3	5/5	5	10.8

TABLE 6

TITRATION OF STRAIN WICKARD IN HAMSTERS, MEDIA PASSAGE NO. 16

Number of Leptospirae per Inoculum		Number of Animals Infected		Number of Deaths		Average Survival Time*	
Whole Culture	Washed Leptospirae	Whole Culture	Washed Leptospirae	Whole Culture	Washed Leptospirae	Whole Culture	Washed Leptospirae
3.06×10^6	7.28×10^5	5/5**	5/5**	5	5	7.2	7.4
3.06×10^5	7.28×10^4	5/5	5/5	5	5	7.8	8.4
3.06×10^4	7.28×10^3	5/5	5/5	5	5	8.8	10.2
3.06×10^3	7.28×10^2	5/5	1/5	5	1	11.0	10.0
3.06×10^2	7.28×10^1	0/5	0/5	0	0	--	--

TABLE 7

TITRATION OF STRAIN WICKARD IN HAMSTERS, MEDIA PASSAGE NO. 20

Number of Leptospirae per Inoculum	Number of Infected Animals	Number of Deaths	Average Survival Time*
7.76×10^6	5/5**	5	6.8
7.76×10^5	5/5	5	7.6
7.76×10^4	5/5	5	8.0
7.76×10^3	2/5	2	11.5
7.76×10^2	1/5	1	12.0

TABLE 8

TITRATION OF STRAIN WICKARD IN HAMSTERS, MEDIA PASSAGE NO. 24

Number of Leptospirae per Inoculum	Number of Infected Animals	Number of Deaths	Average Survival Time*
8.58×10^6	5/5**	5	5.6
8.58×10^5	5/5	5	8.6
8.58×10^4	0/5	0	--
8.58×10^3	0/5	0	--
8.58×10^2	0/5	0	--

TABLE 9

TITRATION OF STRAIN WICKARD IN GUINEA PIGS, MEDIA PASSAGES 1, 4, 8

Passage 1		Passage 4		Passage 8	
Number of Leptospirae per Inoculum	Number of Animals Infected	Number of Leptospirae per Inoculum	Number of Animals Infected	Number of Leptospirae per Inoculum	Number of Animals Infected
1.22×10^4	5/5*	1.02×10^4	3/5*	7.46×10^6	5/5*
1.22×10^3	5/5	1.02×10^3	0/5	7.46×10^5	5/5
1.22×10^2	5/5	1.02×10^2	0/5	7.46×10^4	0/5
1.22×10^1	3/5	1.02×10^1	0/5	7.46×10^3	0/5
1.22	0/5	1.02	0/5	7.46×10^2	0/5

TABLE 10

TITRATION OF STRAIN OHIO IN HAMSTERS,
MEDIA PASSAGE NO. 1

Number of Leptospirae per Inoculum	Number of Animals Infected	Number of Deaths	Average Survival Time*
2.08×10^4	5/5**	5	6.5
2.08×10^3	5/5	5	7.5
2.08×10^2	5/5	5	8.8
2.08×10^1	5/5	5	9.6
2.08	4/5	4	11.5

TABLE 11

TITRATION OF STRAIN OHIO IN HAMSTERS,
MEDIA PASSAGE NO. 4

Number of Leptospirae per Inoculum	Number of Animals Infected	Number of Deaths	Average Survival Time*
4.76×10^4	5/5**	5	7.0
4.76×10^3	0/5	0	--
4.76×10^2	0/5	0	--
4.76×10^1	0/5	0	--
4.76	0/5	0	--

TABLE 12

TITRATION OF STRAIN OHIO IN HAMSTERS,
MEDIA PASSAGE NO. 7

Number of Leptospirae per Inoculum	Number of Animals Infected	Number of Deaths	Average Survival Time*
3.63×10^7	5/5**	5	4.8
3.63×10^6	5/5	5	6.0
3.63×10^5	5/5	5	6.6
3.63×10^4	5/5	5	6.6
3.63×10^3	1/5	1	8.0

TABLE 13

TITRATION OF STRAIN OHIO IN HAMSTERS,
MEDIA PASSAGE NO. 11

Number of Leptospirae per Inoculum	Number of Animals Infected	Number of Deaths	Average Survival Time*
2.00×10^5	5/5**	5	6.2
2.00×10^4	5/5	5	7.4
2.00×10^3	5/5	5	7.8
2.00×10^2	5/5	5	9.2
2.00×10^1	2/5	2	10.5

TABLE 14

TITRATION OF STRAIN OHIO IN GUINEA PIGS,
MEDIA PASSAGES 4 AND 11

Passage 4		Passage 11	
Number of Leptospirae per Inoculum	Number of Animals Infected	Number of Leptospirae per Inoculum	Number of Animals Infected
4.76×10^4	5/5***	2.00×10^5	5/5***
4.76×10^3	0/5	2.00×10^4	5/5
4.76×10^2	1/5	2.00×10^3	5/5
4.76×10^1	0/5	2.00×10^2	5/5
4.76	0/5	2.00×10^1	1/5

TABLE 15

LD_{50} AND ID_{50} VALUES FOR TITRATIONS OF WICKARD AND OHIO STRAINS

Strain	Media Passage	Animal Used for Titration	LD_{50} Value	ID_{50} Value
Wickard	1	Hamster	$>10^5$	3.8
"	4	"	1,509	1,509
"	8	"	235,885	235,885
"	10	"	24,500	16,949
"	12	"	709*	709*
"	16	"	968	968
"	16 (washed cells)	"	1,716	1,716
"	20	"	7,760	7,760
"	24	"	271,200	271,200
"	4 (repeat)	"	1,600	1,212
"	8 (repeat)	"	1,202	261*
"	1	Guinea pig	--	8.2
"	4	"	--	6,323
"	8	"	--	235,885
Ohio	1	Hamster	0.9*	0.9*
"	4	"	15,051	15,051
"	7	"	8,607	8,607
"	11	"	29	29
"	4	Guinea pig	--	15,051
"	11	"	--	47

*50 percent endpoint not reached. These values represent the highest possible value which could be calculated from the data.

TABLE 16

THE EFFECT OF THE AGE OF HAMSTERS ON SUSCEPTIBILITY TO L. POMONA (WICKARD)

Dilution of Infected Guinea Pig Blood	Number of Four Week Old Hamsters Infected	Number of Eight Week Old Hamsters Infected
10^{-1}	5/5*	5/5*
10^{-2}	5/5	5/5
10^{-3}	4/5	5/5
10^{-4}	0/5	2/5
10^{-5}	0/5	0/5

Dilution of Infected Guinea Pig Blood	Number of Four Week Old Hamsters Infected	Number of Fifteen Week Old Hamsters Infected
10^{-2}	5/5*	5/5*
10^{-3}	3/3**	5/5
10^{-4}	1/5	1/5
10^{-5}	0/5	0/5
10^{-6}	0/5	0/5

*Numerator = number of hamsters infected;
Denominator = number of hamsters inoculated.

**Two hamsters escaped from the cage. Thus only 3 hamsters remained for infectivity determinations.

TABLE 17

GROWTH OF L. POMONA IN VITRO AFTER 1, 4 AND 8 MEDIA PASSAGES

Passage 1		Passage 4		Passage 8	
Number of Leptospirae per Inoculum	Number of Tubes Showing Growth	Number of Leptospirae per Inoculum	Number of Tubes Showing Growth	Number of Leptospirae per Inoculum	Number of Tubes Showing Growth
1.22×10^4	7/7*	8.6×10^4	5/5*	7.46×10^4	5/5*
1.22×10^3	7/7	8.6×10^3	5/5	7.46×10^3	5/5
1.22×10^2	7/7	8.6×10^2	5/5	7.46×10^2	5/5
1.22×10^1	7/7	8.6×10^1	5/5	7.46×10^1	5/5
122	2/7	8.6	1/5	7.46	0/5

*Numerator = number of tubes with growth;
Denominator = number of tubes inoculated.

TABLE 18

IN VIVO ADHESION OF STRAIN WICLARD AND ERYTHROCYTES

Blood Material	Dilution of Inoculum	Number of Infected Hamsters	Number of Culture Tubes with Growth	Number of Control Culture Tubes with Growth
Whole Blood	10^{-2}	5/5*	5/5**	5/5**
"	10^{-3}	1/5	5/5	5/5
"	10^{-4}	0/5	5/5	4/5
"	10^{-5}	0/5	0/5	--
Plasma	10^{-1}	5/5	5/5	--
"	10^{-2}	4/5	5/5	5/5
"	10^{-3}	0/5	0/5	5/5
"	10^{-4}	0/5	0/5	2/5
Erythrocytes	10^{-1}	5/5	5/5	--
"	10^{-2}	4/5	5/5	5/5
"	10^{-3}	0/5	2/5	5/5
"	10^{-4}	0/5	0/5	2/5

*Numerator = number of hamsters infected;
Denominator = number of hamsters inoculated.

**Numerator = number of tubes with growth;
Denominator = number of tubes inoculated.

--Not determined.

TABLE 19

IN VITRO ADHESION OF LEPTOSPIRAE AND ERYTHROCYTES AFTER INCUBATION AT 37 C FOR THIRTY MINUTES

Tube No.	Components*	Number of Leptospirae per cc of Supernate[1]
1	0.9 cc saline	2.6×10^7
2	0.9 cc whole blood**	3.2×10^7
3	0.1 cc RBC** + 0.8 cc saline	2.6×10^7
4	0.1 cc RBC** + 0.8 cc inactivated normal sera***	2.4×10^7
5	0.1 cc RBC** + 0.1 cc antisera**** + 0.7 cc inactivated normal sera***	1.0×10^7
6	0.1 cc RBC** + 0.1 cc antisera**** + 0.7 cc normal sera**	0.9×10^7

*0.1 cc of a culture of L. pomona (Wickard) was added to each tube.

**Normal ovine blood, RBC's and serum.

***Normal ovine sera inactivated at 55 C for 30 minutes.

****Ovine sera containing L. pomona antibody at a titer of 10^{-5}.

[1]Numbers determined by Petroff-Hauser counting chamber.

TABLE 20

THE HEMOLYSIS OF SHEEP ERYTHROCYTES BY VARIOUS CULTURES OF *L. POMONA*

Culture	Age of Culture	Number of Leptospirae in Culture	LD_{50} of Culture*	Percent Hemolysis of Culture Dilutions										Culture Dilution Producing 50 Percent Hemolysis
				1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	
[1]Strain Wickard from infected cow in media 3 yrs	12 days	1.3×10^8/cc	--	90	76	68	51	20	10	0	0	0	0	1:17
[2]Strain Wickard from guinea pig media passage 3	14 days	1.1×10^8/cc	-	90	84	75	74	65	35	11	0	0	0	1:48
[3]Strain Wickard from sheep media passage 3	13 days	1.1×10^8/cc	5.0×10^3	90	71	63	51	22	8	0	0	0	0	1:15
[4]Strain Wickard from sheep media passage 4	14 days	--	1.5×10^3	100	90	90	90	90	55	29	10	0	0	1:80
[5]Strain Wickard from sheep media passage 9	10 days	1.9×10^8/cc	1.0×10^3	90	74	72	69	55	26	8	0	0	0	1:35
[6]Strain Wickard from sheep media passage 10	13 days	1.0×10^8/cc	1.0×10^3	100	90	80	72	48	16	0	0	0	0	1:30
[7]Strain Wickard from sheep media passage 25	10 days	1.9×10^8/cc	4.0×10^5	100	100	100	90	85	85	63	51	22	13	1:230

TABLE 20 - continued

Culture	Age of Culture	Number of Leptospirae in Culture	LD_{50} of Culture*	Percent Hemolysis of Culture Dilutions										Culture Dilution Producing 50 Percent Hemolysis
				1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	
[8]Strain Ohio from guinea pig media passage 4	13 days	0.9×10^8/cc	1.5×10^4	90	90	80	75	60	16	0	0	0	0	1:57
[9]Strain Ohio from guinea pig media passage 6	13 days	1.4×10^8/cc	1.0×10^4	100	85	71	65	51	11	0	0	0	0	1:28
[10]Strain Ohio from guinea pig media passage 8	10 days	1.6×10^8/cc	3.3×10^3	90	85	80	65	58	22	0	0	0	0	1:31

*Extrapolated from Figure 1.

--Not determined.

TABLE 21

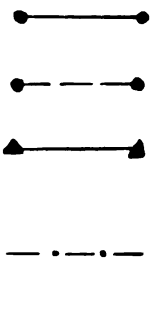
THE HEMOLYSIS OF ERYTHROCYTES FROM VARIOUS ANIMALS BY L. PORCINA

Culture* No.	Source of Erythrocytes	Percent Hemolysis of Culture Dilutions			
		1:2	1:4	1:8	1:16
7	Cow	100	100	90	85
"	Rabbit	51	40	31	10
"	Hamster	24	15	10	0
"	Guinea pig	0	0	0	0
1	Cow	85	75	66	41
"	Rabbit	0	0	0	0
"	Hamster	0	0	0	0
"	Guinea pig	0	0	0	0
2	Cow	90	85	85	80
"	Rabbit	20	0	0	0
"	Hamster	15	10	0	0
"	Guinea pig	0	0	0	0
8	Cow	90	90	90	75
"	Rabbit	17	0	0	0
"	Hamster	17	10	0	0
"	Guinea pig	0	0	0	0
3	Hamster	10	0	0	0
4	Hamster	20	11	0	0
6	Hamster	16	0	0	0
9	Hamster	10	0	0	0
10	Hamster	10	0	0	0

*Numbers refer to cultures in Table 20.

FIGURES

Figure 1.



Strain Wickard

Strain Ohio

Repeated titrations of strain Wickard

Estimation of virulence since the first medium passage of strain Wickard was not lethal

Figure 2. Line A = First medium passage of strain Ohio. $LD_{50} = 0.9$

Line B = Sixteenth medium passage of strain Wickard. $LD_{50} = 10^3$

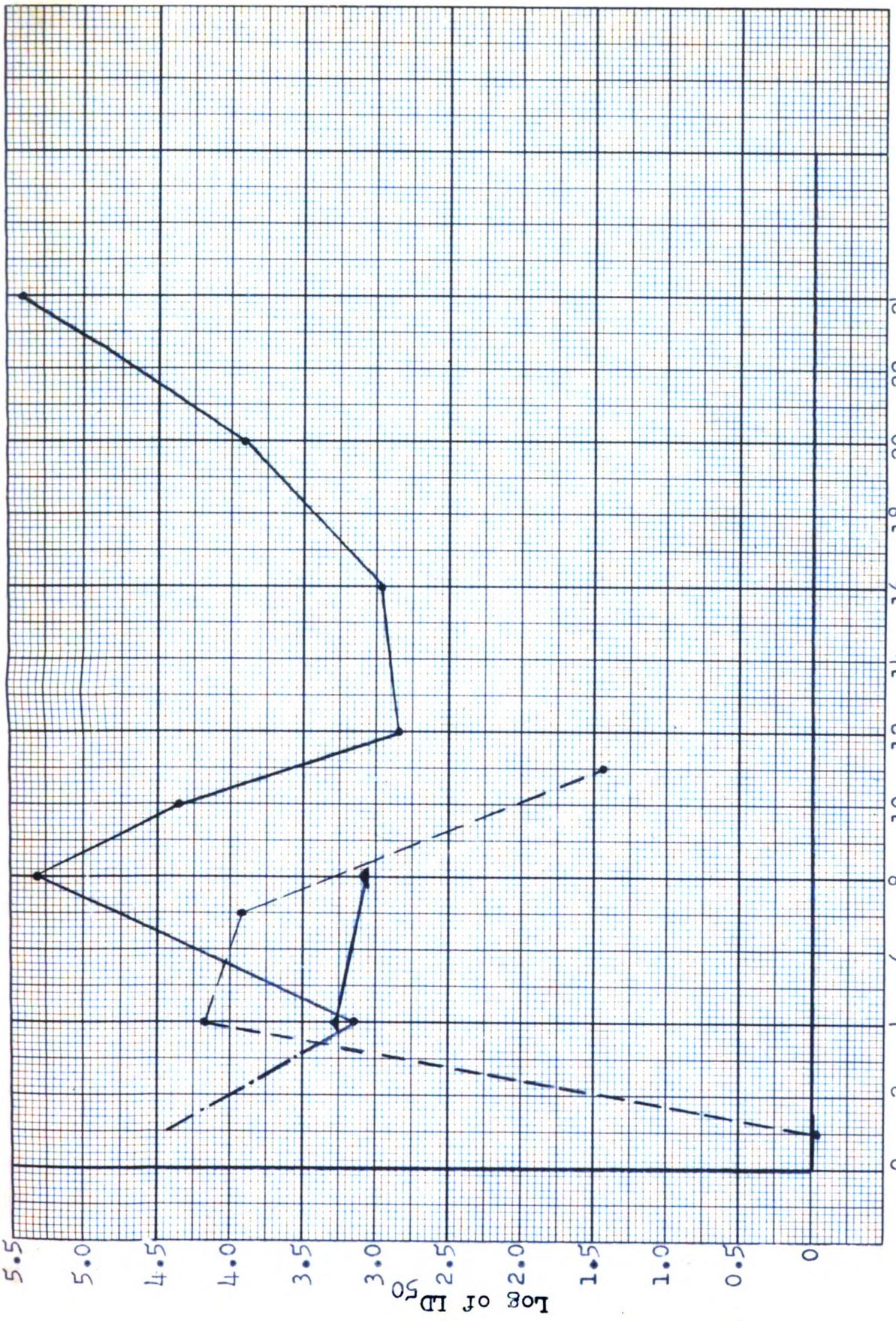


Fig. 1. The effect of passage in media on the virulence of L. pomona for hamsters.

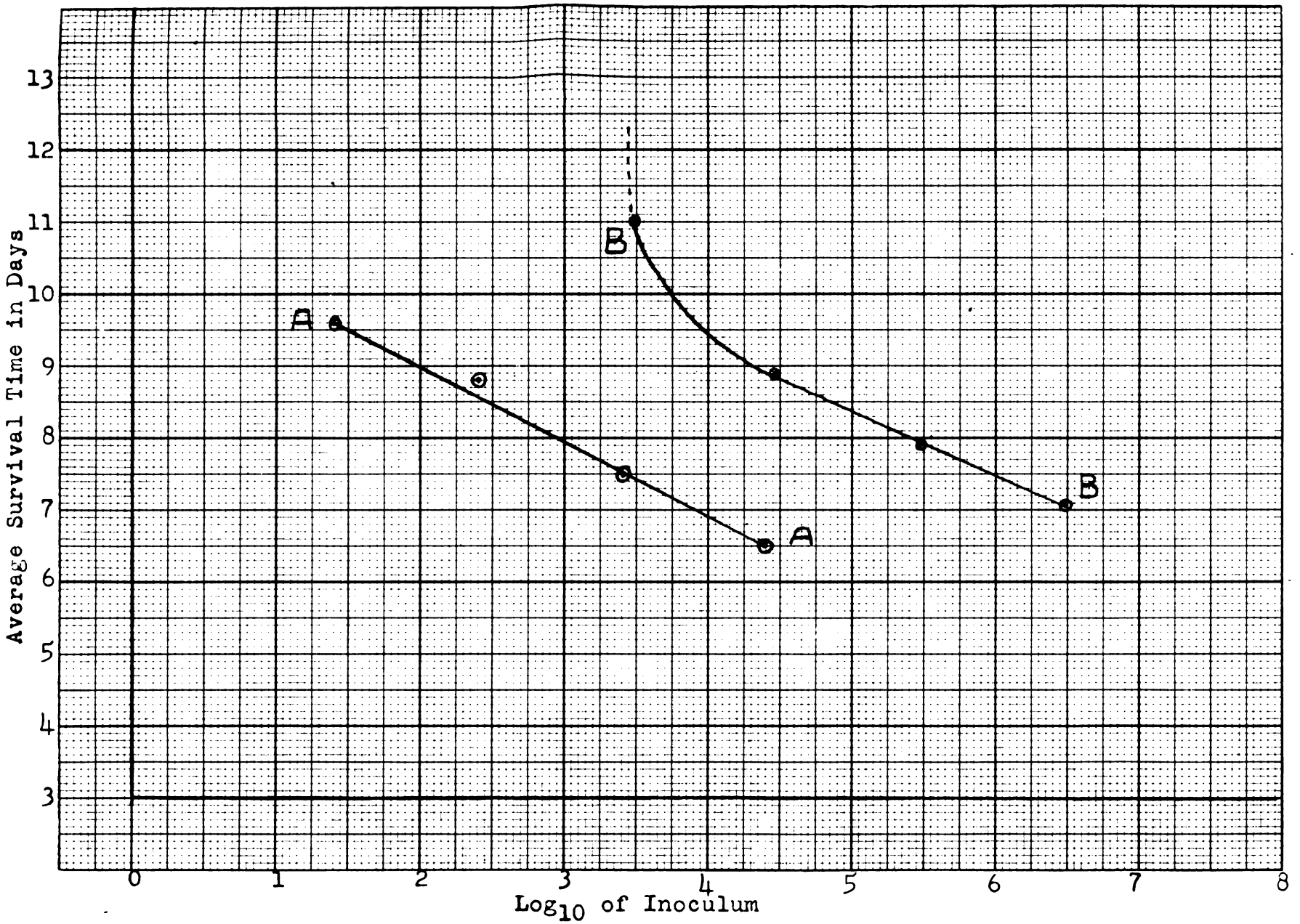


Fig. 2. The effect of the size of the inoculum on the survival time of hamsters.

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