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THE IMMUNOLOGICAL REACTIVITY OF SEVERAL SPECIES OF ANIMALS TO LEPTOSPIRA POMONA AND FRACTIONS THEREOF

by

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ABSTRACT

This investigation had two objectives: (a) to determine the relative susceptibility of several animal species to L. pomona infection and (b) to study the immunological reactivity of guinea pigs and calves to killed suspensions of the organism and fractions thereof. An examination was also made into some of the methods presently used to detect infection or immunity.

The relative susceptibility of several animal species to L. pomona infection was compared with that of guinea pigs. Therefore it was first necessary to assay the infectivity of the organism in this species. Bacterial counts were made of young leptospiral culture which was then diluted to provide appropriate dose levels. Sixty guinea pigs in groups of four animals each were injected with infective material ranging down from 100,000 to 13 organisms per inoculum. The level of 50 leptospirae per inoculum was selected as the guinea pig infective dose since it was the lowest in which all animals in the group became infected and showed a regular response. The larger doses of leptospirae did not produce more severe disease as judged by the temperature response. In fact, disease appeared to be less severe at the higher dose levels but occurred sooner after exposure. Ultimate agglutination-lysis titres were not affected by the number of organisms in the infective dose.

Multiples of the guinea pig infective dose proportional to body weight of the species were used in an attempt to infect young guinea pigs, hamsters, rabbits, calves and pigs. Hamsters, rabbits and

calves could be infected in this way but swine did not become infected with the appropriate dose level nor with 100 times that amount.

The immunological response of guinea pigs to seven different killed suspensions of leptospirae was compared. Antigenic material was injected followed two weeks later by challenge material of known infectivity. The antigens varied widely in their ability to stimulate production of protective antibody. One actually appeared to accelerate infection.

The best and the poorest of these seven antigens were used in calves and their immunity subsequently challenged. Following challenge serum antibodies were demonstrable in the calves by the agglutination-lysis test but the two groups of animals could not be differentiated clearly. The complement-fixation test showed high serum antibody titres in the animals which had received the poor antigen and none in those which received the good antigen. This was interpreted as meaning that the former group had lacked protective antibody and had developed infection after challenge. Therefore, the results in calves paralleled those in guinea pigs with respect to the antigenicity of the immunological agents.

Water-soluble and water-insoluble fractions of alcoholic extracts of L. pomona organisms were prepared. Tests in guinea pigs failed to reveal antigenicity in vivo although the agents have been shown to be reactive in vitro. The water-insoluble fraction was a satisfactory complement-fixing antigen throughout these studies.

Comparisons were made of tests used for the demonstration of

leptospirae in tissues and body fluids. Cultural techniques were found reliable in these studies where specimens were not otherwise contaminated. Dark-field examination was moderately successful in certain kinds of material. The fluorescent technique yielded positive findings only when leptospirae were relatively numerous in the specimen.

Studies of three serological tests for leptospirosis suggested that the agglutination-lysis reaction was the most sensitive indicator of an animal's experience with a specific leptospiral serotype. The complement-fixing antibodies tended to appear only during or for a short time after active infection and therefore provided a better index of present or recent infection. The plate-agglutination test results approximated those of the complement-fixation test.

Agglutination-lysis antibody titres of kidney suspensions were not of significent magnitude to suggest renal antibody production. The low titres obtained could be accounted for by residual serum in the tissue.

The mechanism of the agglutination-lysis test was investigated. It was shown that complement had no effect upon the titres obtained. It was also indicated that while lysis appeared to take place this in reality did not occur.

Leptospirosis caused by L. pomona is a serious public health problem in some countries but the condition has not been reported in man in Canada. It is likely that a mild form of the disease exists because there is a significant incidence of infection in domestic animals and wildlife. Fundamental knowledge of the immunological response of animals to leptospirosis should provide a better basis for understanding the disease in man.

THE IMMUNOLOGICAL REACTIVITY OF SEVERAL SPECIES OF ANIMALS TO LEPTOSPIRA POMONA AND FRACTIONS THEREOF

INTRODUCTION

Pathogenic "spirochaeta" were first described in 1907 by Stimson (1) who saw them in sections of kidney tissue from a patient believed to have died of yellow fever. The sections were stained by Levadite's silver impregnation method. Interest was not aroused until 1914 when Inada and Ido (2) in Japan demonstrated "spirochaeta" in liver tissues of guinea pigs which had been injected with blood from cases of Weil's disease. They called the organism Spirochaeta icterohaemorrhagiae. In 1917 Noguchi (3) suggested the generic name leptospira (lepto-fine; spira-coil). Since that time over 60 other serotypes of leptospirae have been identified from all parts of the world. In addition to Weil's disease or haemorrhagic jaundice, leptospirae have been found responsible for a variety of human conditions such as seven-day fever, swineherd disease, rice field fever, canefield fever, autumn fever, canicola fever and Fort Bragg fever.

During 1937 investigation by Clayton et al (4) of a case of "seven day fever" in a dairy farmer in the Pomona district of Queensland, Australia, resulted in the isolation of a leptospira. The organism was described by Derrick (5) in 1942 and was given the name Leptospira pomona. Mochtar (6) isolated this serotype from pigs in Batavia, Java, in 1940. In 1944 Gsell (7) in Switzerland showed it

was also the cause of "swineherd disease". On this continent leptospira-like structures were found by Jungherr (8) in liver, kidney and mesenteric lymph node tissues of three cattle that died in Connecticut during 1943 and 1944 but he was unable to isolate the organism. Baker and Little (9) published a complete report of their isolation of leptospirae from abnormal milk of dairy cattle and of their transmission experiments. They did not identify the serotype however. This was done in 1950 by Gochenour (10) who identified it as L. pomona.

Presumptive evidence of the occurrence of leptospirosis due to L. pomona in Canada was found by Smith and Perry (11) who demonstrated leptospirae in histopathological sections from a young bull in 1952. In 1956 a serological survey by Moore and Rice (12) of approximately 1,000 cattle from Ontario and Quebec showed over six per cent positive reactions in the complement-fixation test. The first actual isolation of L. pomona in Canada was made in 1956 by Barnum and Grinyer (13) who recovered it from a three-week old calf. Other studies indicated L. pomona infection to occur commonly in Canadian cattle (14, 15) but this serotype has not yet been reported in man in this country.

Since the discovery of L. icterohaemorrhagiae, medical interest in the leptospirae has been maintained in Europe and Asia. In these areas leptospirosis has been and still is a serious public health problem. In contrast, the medical profession on the North American continent has tended to regard leptospirosis as an exotic disease or as a tropical condition. It is only during the last decade that infection in domestic animals has attracted attention and stimulated research. Much of this investigation has been devoted to epidemiological

studies, surveys of incidence and the reporting of new serotypes.

The usual bacteriological procedures are not applicable to leptospirae and the methods which prove appropriate require the use of experimental animals. Many problems connected with variations in susceptibility of the host to infection, with the mechanisms of host resistance, with the chemistry of leptospirae and with identification of their antigenic components remain to be investigated. The work to be presented in this thesis involves the study of several of these -- being concerned with three main points:

(a) the relative susceptibility of various species of animals to L. pomona infection,

(b) the immunological reactivity of several species of animals to killed suspensions of L. pomona and fractions thereof,

(c) the investigation of some methods presently used to determine infection and immunity.

LITERATURE REVIEW

CLASSIFICATION AND DESCRIPTION OF LEPTOSPIRAE

Breed, Murray and Smith (16) have classified leptospirae in the order Spirochaetales because of the coiled morphology of the organism, and in the family Treponemataceae because the spirals are small and there is no obvious protoplasmic structure. In this family leptospirae are grouped with the genera Borrelia and Treponema. In spite of their resemblances there are characteristics which differentiate them. Borrelia is the only member which stains easily with aniline dye. Leptospira are then differentiated from Treponema because the former is an aerobe whilst

the latter is an anaerobe.

As mentioned in the introduction, the genus Leptospira was suggested in 1917 by Noguchi (3) who was studying "Spirochaeta icterohaemorrhagiae". In 1918 he made a detailed comparative study of the spiral organisms and illustrated his observations with drawings and 112 excellent photomicrographs (17). Based largely on Noguchi's work Breed, Murray and Smith (16) described leptospirae as finely coiled organisms six to 20 microns in length, the spirals measuring 0.3 microns in depth and 0.4 to 0.5 microns in amplitude. In liquid media either one or both ends of the cells are bent into a semicircular hook, each involving 1/10 to 1/8 of the organism. Spinning movements occur in liquid media and vermiform movements forward or backward in semisolid agar. In living preparations the cells are observed most clearly with dark-field and much less clearly with phase-contrast microscopy; they are not visible with ordinary illumination. The organisms stain with difficulty except with Giemsa's stain and the silver impregnation method. An axial filament can be demonstrated by electron microscopy. Leptospirae require oxygen for growth.

It is emphasized that leptospirae cannot be studied by the usual bacteriological methods and therefore it has proven difficult to apply the species concept to their classification. However, similarities and differences in antigenic constitution have allowed arbitrary grouping and subgrouping of isolates into serogroups and serotypes. The system was proposed by Wolff and Broom (16) in 1954. Strains which were antigenically distinct on the basis of agglutination and cross-absorption tests were called serotypes. Serotypes having

major antigens in common were grouped into serogroups.

PUBLIC HEALTH SIGNIFICANCE OF LEPTOSPIROSIS

L. Pomona infection in man is commonly known as swineherd disease or as pomona fever. Clayton (4) described the disease and stated that it consisted of pyrexia of about seven or eight day's duration, the illness usually being mild, with no cases of jaundice or fatalities. Woodward (18) stated that there may be a relatively high incidence of aseptic-type meningitis. In general the disease is likely to escape detection since it often resembles influenza. Sex and age incidences were studied by Gsell (19) who found the proportion of cases of infection was greater in males than in females. There was a sharp rise in incidence to a peak in the 20 to 29 years age group followed by a rapid decline. McCrumb (20) is of the opinion that infection is a function of man's habits and has nothing to do with predilections of the leptospirae. Pomona fever occurs largely in occupational groups having close contact with cattle and swine such as farmers (4, 7), veterinarians (21,22) and abattoir workers (23). Schaeffer (24) reported a large outbreak involving over 50 young people who had been swimming in a pool which was fed by a stream draining an infected hog farm. Reviews by Alston and Broom (6) and by Steele (25) indicate the disease to be widely disseminated throughout the world and to be a serious public health problem. Derrick (5) has stated it to be an important cause of human illness in Australia.

VETERINARY SIGNIFICANCE OF LEPTOSPIROSIS

It is generally agreed that leptospirosis is primarily a disease of rodents and depends upon them for survival and that man and

domestic animals become infected by accident (20). However, L. pomona has become well adapted to swine and these animals are considered to be the main reservoir of infection of this serotype (26, 27). In a random survey of 285 market hogs in the United States, Burnstein and Baker (28) found 22 per cent to be serologically positive. Morse (29) stated that losses occur due to late abortion, as high as ten to 50 per cent of infected sows in a herd aborting, with the average litter size of other sows in the herd reduced from a normal of ten piglets to approximately three. Burnstein and Baker (28) on the other hand from their studies of the disease in swine concluded that infection is usually benign or inapparent. The animals continued to eat and behave normally but a brief temperature rise occurred between the 7th and 14th post-exposure days. The fact that swine may shed leptospirae in the urine for a very long period constituted a serious problem. Burnstein and Baker (28) observed shedding for over 159 days, while Reinhard (30) stated that shedding may occur for over six months. Bohl (31) quoted Schnid and Giovanella as having observed the passing of leptospirae in swine urine for more than a year.

Leptospirosis is a disease of particular importance in cattle. Serological surveys (27,29) in the United States have indicated incidences of past and present experience in cattle to vary from five to 50 per cent in different parts of the country. Moore and Rice (12) using the complement-fixation test found the presence of antibodies in 6.3 per cent of cattle sera from Ontario and Quebec. In another survey from the same area using the agglutination-lysis test Boulanger and

Smith (14) found an incidence of 8.1 per cent reactions in 2695 cattle tested. Of the 113 herds represented, 60.1 per cent contained reactors. The disease in adult cattle may take a mild inapparent form or may be more severe showing temperatures up to 105.5°F., depression, anorexia and even diarrhea. Icterus and haemoglobinuria are rare but abortions in pregnant cattle and the secretion of abnormal milk are fairly frequent (32). In replies to a questionnaire sent to veterinarians in Illinois, Bryan (33) found a reported incidence of 17 per cent abortions and 9.6 per cent placental retentions among 2,787 affected cattle; in 39 per cent of the infected herds there had been an increase in repeat breedings or animals sold as non-breeders. In young calves leptospirosis is much more severe, often resulting in high temperature, icterus, haemoglobinuria and death. Those which recover may remain stunted (34).

Horses are also susceptible to leptospirosis and Jackson (35) has shown that it may produce abortion in mares.

There are few reports in the literature relative to the incidence or importance of L. pomona infection in sheep. Hartley (36) in New Zealand recorded the first two outbreaks in that country which occurred in 1950 and 1951 respectively. Webster and Reynolds (37), also in New Zealand, reported an outbreak in a 400 ewe stud flock and their lambs. Morbidity reached 100 per cent in the lambs and mortality 18 per cent. Many of the survivors were severely affected. In the adults the disease was mild and often subclinical. In the United States, Beamer et al (38) observed a severe outbreak in 108 young ewes, in which the illness was followed in about a week by abortions. Often the ewe became progressively weaker and died. Others (39,40,41) have studied the experimental infection in sheep

and concluded that in the United States these animals play a minor role in the transmission of L. pomona. Canadian literature contains no reference to leptospirosis of sheep but it is probable that no effort has been made to determine its presence.

L. pomona infection may also be found in dogs (42-45) and cats (46) but, contrary to what one would expect, the disease is rare.

While it has been universally accepted that swine are the reservoir of infection with L. pomona it is becoming increasingly evident that a higher percentage of outbreaks in cattle and sheep cannot be traced to swine (47, 48, 49). Observations such as these have encouraged experiments on infectivity and surveys to determine if infection existed in wildlife (47, 48, 50-57). Large scale studies of this nature are obviously difficult to conduct and surveys so far suffer from sampling being too small to give significant figures for incidence of infection. However, natural infection has been found in a large number of wild mammals such as deer, hedgehog, raccoon, fox, skunk, opossum, rat and mouse. The hedgehog may be an important reservoir for L. pomona in New Zealand according to Webster (47, 48). In Ohio a survey by Goldstein et al (54) revealed significant agglutination-lysis antibody titres using L. pomona antigen in the sera of 43 of 226 deer, 16 of 70 raccoon, 14 of 55 foxes and six of 11 skunks. McKiel et al (58) made a survey of rats and other small animals in Eastern Canada using cultural methods and the agglutination-lysis test of the sera. The only serotype isolated from 426 rats in the study was L. icterohaemorrhagiae. However, one rat trapped in the vicinity of a piggery where swine were known to be carriers of L. pomona had a titre of 1:3200 for this serotype. A guinea pig inoculated

with tissue from another rat trapped elsewhere developed a titre of 1:100 against L. pomona. Evidence of infection with L. pomona was also found in two of 27 ground hogs and in two of ten skunks. Negative findings were obtained in five cats, two snowshoe hares, six meadow voles, seven grey squirrels and four chipmunks.

THE SUSCEPTIBILITY OF VARIOUS ANIMAL SPECIES TO LEPTOSPIROSIS

While the classification of resistance as "natural" and "acquired" is convenient for an orderly presentation it must be borne in mind that there is no sharp line of division between the two. For example, it will be apparent from references already presented that L. pomona often produces severe disease and death in young calves. Severity rapidly decreases as age increases and illness may be slight or inapparent in the adult. This may simply reflect more prompt antibody response in older animals rather than the presence of a natural barrier, unless increased ability to react is itself considered a natural barrier.

NATURAL RESISTANCE

Literature already cited indicates a wide variety of animal species to be susceptible to infection with L. pomona. No reference has been found to show absolute natural resistance in any mammal. Variations in incidence of infection in different species as revealed by surveys do not necessarily indicate relative differences in susceptibility. They may only be a function of the environmental factors and opportunity for infection.

Controlled experiments into the relative susceptibilities of

the large domestic animals have not been reported. Such work has undoubtedly been discouraged by the expensive nature of these studies. However, the desire for suitable inexpensive laboratory animals for the isolation of leptospirae from contaminated material and for experimental work has stimulated such investigations.

Guinea pigs were used in the original isolations of L. icterohaemorrhagiae by Inada and Ido (2) and in the studies by Noguchi (3). The first demonstration of L. pomona was also made in guinea pigs by Derrick (4) from human blood submitted to his laboratory by Clayton. Guinea pigs have always been considered to be quite susceptible to leptospirosis and are probably the most widely used experimental animals for studies of this disease although death of guinea pigs infected with L. pomona is rarely observed. Infection is detected by a temperature rise, by demonstration of leptospirae in the blood at the acute stage of disease and by the appearance of serum antibodies after the animal has recovered.

Hamsters were used by Randall and Cooper (59) in their experiments for the isolation of L. canicola and L. icterohaemorrhagiae because they found this animal species to be more susceptible to L. canicola infection than guinea pigs or rats. Morton (60) from studies of the infectivity of these two serotypes for hamsters, concluded that the hamster is a useful experimental animal. The virulence of L. pomona for hamsters has been investigated by Hamdy and Ferguson (61) who found the animals were readily infected but mortality was low.

White mice were used by Larson (62) who found them extremely

susceptible to L. icterohaemorrhagiae infection. However, Stavitsky and Green (63) with a different strain of white mice and Larson's culture of leptospira failed to produce fatal infection. This led to the conclusion that there are differences in susceptibility within strains of mice. To explain this Stavitsky and Green postulated a greater rate of production of lytic antibodies in the resistant strain than in the more susceptible strains. White mice have not received acceptance by those working with L. pomona. Their small size does not permit inoculation of large amounts of contaminated material for isolation purposes and the yield of serum is small for serological studies.

Roberts and Turner (64) made a comparative study of the infectivity of L. pomona in adult chinchilla, young guinea pigs and hamsters. They found the chinchilla to be highly susceptible and all animals died whereas the guinea pigs and hamsters survived. They suggested the chinchilla would be useful experimental animals.

Experimental studies by Webster (47, 48) using young hedgehogs revealed them to be very susceptible to L. pomona and infection usually resulted in death. Older animals become carriers. The author suggested hedgehogs as experimental animals.

Hoag et al (65) produced experimental infection with L. pomona in two-day old chicks. There was no clinical illness but leptospiremia commenced about the fourth post-inoculation day and was of five days duration. The organism could be recovered from liver and kidney tissue between the ninth and fourteenth days. Howarth and Reina-Guerra (66) also used two-day chicks and found that irradiation enhanced their susceptibility.

No symptons were shown by the chicks. While leptospiremia could be demonstrated, leptospiruria could not.

A comparative study of the susceptibilities of guinea pigs, hamsters, white mice and two-day old chicks was made by Ringen and Okazaki (67) in 1956. A strain of L. pomona which was considered to be stabilized in culture medium was used as the infective agent. Bacterial counts were made and groups of the experimental animals were inoculated intraperitoneally with varying dosages from 100 to 14000 leptospirae. It was found that guinea pigs and white mice were about equally susceptible to infection and that hamsters were considerably more resistant. Chicks were highly resistant.

In addition to species differences in susceptibility, strain differences have been noted. In this regard Stavitsky's findings (63) in white mice have already been cited. A similar observation was made with respect to breeds of cattle by Morter and Morse (68) studying L. pomona infection in calves. Hereford calves appeared to be considerably more susceptible to infection than Holsteins.

In addition to species and strain differences individual variations in susceptibility to leptospiral infection have been observed. Noguchi (69) commented on this during the course of his studies with L. icteroides which he believed to be the cause of yellow fever. His observations are quoted as follows "Such refractory guinea pigs are rarely met with, but the fact that there exist certain unusually resistant individuals is of great interest. It has also been found that there are extremely susceptible individuals which respond to infection with an

attenuated culture which no longer attacks the average guinea pig".

Age was found to make considerable difference in the susceptibility of animals to leptospiral infection. This factor was emphasized by Larson (62) who found that the high susceptibility of his strain of white mice fell rapidly after the animals were 21 days of age. Babudieri (70) advised the use of young guinea pigs because of their greater susceptibility than that of older animals. Morton (60) cautioned that hamsters over five weeks of age should not be used. Webster (47,48) found young hedgehogs to be much more severely affected than older animals. Observations of naturally occurring outbreaks in herds of cattle and flocks of sheep have shown the younger animals to be most severely affected.

There is little evidence to show that a significant difference exists between the susceptibilities of the sexes to leptospiral infection. Smith et al (71) studied natural infection in rats in Malaya by several leptospiral serotypes. They found a general tendency for females to be more frequently infected than males but significant differences were found in only two species of rats. They speculated that the higher incidence in females may have been due to the passage of leptospirae in the urine of males to the mucous membranes of the female during coitus.

ACQUIRED RESISTANCE

The mechanism of resistance to spirochaetal infection has been studied by Stavitsky (72). On the basis of his own work and of his review of the literature he does not consider the usual barriers to infection to be of significance in leptospirosis. Neither the peripheral barriers nor the normal constitutents of the blood, including the leucocytes, appear

to be capable of effective defence against the disease. However, following exposure to the organism, development of specific antibodies occurs in the animal body. It is only with the development of these antibodies that effective resistance is achieved. Olitzky (73) observed wide variation in the abilities of cattle to produce agglutinins in their sera. Brunner and Meyer (74) noted in their experiments with L. canicola and L. icterohaemorrhagiae in dogs that the control animals which died of infection, had failed to respond serologically like the survivors. Variation in susceptibility of species, strains, individuals and age groups might then be explained by the promptness of antibody production and upon the level obtained. This may vary genetically and with the age of the animal.

Following infection, effective immunity of long duration is produced in the animal against the serotype which stimulated it. York and Brueckner (75) have stated that such cattle are probably resistant for life although supporting evidence was not produced. Webster and Reynolds (37) working with sheep in New Zealand also observed that recovered animals are highly resistant to reinfection. Morter et al (76) attempted to reinfect sows ten to 14 months following initial experimental infection. The sows were pregnant but no abortion or other evidence of infection occurred. Challenge produced only a transient rise in antibody titre and the sows were considered to have been protected. On the other hand Olitzki et al (73), working in calves with a serotype identified only as "bovine strain", noted that recovery from infection did not connote solid protection against reintroduction of the same serotype.

Interest in artificially induced resistance to leptospirosis

has been stimulated by the need for an immunogenic agent. Much work has been directed to the development and evaluation of such agents for use in cattle and swine. In the search for an antigen which would stimulate the highest possible degree of protection numerous techniques have been employed.

Olitzki et al (73) working in Palestine attempted to stimulate protective antibodies in calves against a highly virulent leptospira identified as "bovine strain". This organism has later been referred to as L. bovis. To do this they infected calves with living culture of L. grippotyphosa which was closely related antigenically to L. bovis but produced only a mild infection in calves. Formalized cultures of L. bovis were also used and results of the two methods compared. It was found that the living antigen stimulated better protection.

It has been noted repeatedly since Noguchi's early studies that leptospirae rapidly loose virulence during successive subculturing in artificial media but York and Baker (77) observed that pathogenicity is maintained when the organisms are cultivated in embryonating eggs. Since it was thought that pathogenicity might have a bearing upon antigenicity, egg propagation of the leptospirae was introduced in production of antigen. The culture used was the original strain of L. pomona isolated in the United States (Cornell T 262) which had been maintained in eggs since that time. The culture was inactivated by freezing and thawing since this technique seemed less likely to affect antigenicity than chemical methods. Effective protection of calves resulted from the inoculation of 5 ml. of the killed antigen subcutaneously.

Brown et al (78) produced an antigen by growing the T262 strain of L. pomona in a liquid medium (Stuarts) and inactivating it with thimersol in a final dilution of 1:10,000. Tests revealed that a single subcutaneous injection of 5 ml. into calves elicited sufficient serum antibody to give good protection.

In New Zealand, Webster and Reynolds (37) maintained their cultures of L. pomona in semi-solid or colloid medium prepared by adding 0.15 per cent agar to the liquid medium. Such medium was thought to be superior to liquid in maintaining virulence. Inmaking their antigen, however, they omitted the agar. The culture was inactivated by heat ($56^{o}C$ for two hours in a water bath) since it had been shown by Ezell et al (79) that leptospira culture liquor contained a heat-stable complement-fixing antigen. Antigenicity was evaluated in sheep which became completely resistant to infection for over 11 months following immunization.

Brunner and Meyer (74) who used lyophilization to kill and concentrate cultures of L. canicola and L. icterohaemorrhagiae found that antigens prepared from L. icterohaemorrhagiae strains were less immunogenic than those prepared with L. canicola. No cross protection was conferred by L. canicola against L. icterohaemorrhagiae infection. Perhaps their most interesting observation was that antigens prepared from less virulent strains of L. canciola were less effective in stimulating protective antibody. It required three times as much antigen from an avirulent strain to afford the same protection as that from a virulent strain. York (80) has commented that the method of propagation of leptospirae is not as important in antigen production as the selection

of a strain which retains antigenicity.

The use of multiple doses of antigen was investigated by Olitzky et al (73), who found that somewhat better although not solid protection was achieved in calves against L. bovis than when single injections were given. Brown et al (81) noted that doubling the usual five to ten ml. dose of antigen administered to horses stimulated higher serum titres which persisted longer. They concluded that the larger doses had stimulated higher immunity.

It is generally recognized that a better immunological response can be stimulated in older animals than in the very young. This has been found to be true in the case of leptospirosis. Gillespie and Kenzy (82)found that calves immunized at one to two months of age acquired less resistance to infection than those immunized at six to eight months.

Brown et al (81, 83) studied the serum antibody titres of cattle and horses immunized against L. pomona infection. Within a week antibody detectable by the agglutination-lysis test appeared and rapidly reached a peak titre which was maintained for about two months. There was then a gradual decline and in most animals antibody could no longer be demonstrated in six months. Bierer et al (84) observed high agglutination-lysis titres in the serum on the 20th to 28th day tests. These had dropped sharply by three to four months and had declined to a low level by five to six months. Even after 13 to 16 months, however, 40 per cent of the 485 cattle tested still had low agglutination-lysis titres. Taylor et al (85) were able to demonstrate serum antibodies against L. pomona for a year after immunization in a herd of 100 cattle.

There is a difference of opinion concerning the relationship between the agglutination-lysis antibody titre of the serum and the degree of protection an animal has against infection. Duration of protection following immunization has been studied by Brown et al (83). They showed that protective antibody persists much longer than the relatively short period during which appreciable amounts of serum antibodies can be demonstrated. Studies by Scheidy (86) in cattle, sheep, swine and horses indicated protection of these animals six months and one year following immunization. Gillespie and Kenzy (82,87) found a correlation between the immunization titre and resistance to challenge infection in young cattle. Brunner and Meyer (74) considered the agglutination-lysis titre a useful measure of the degree of protection conferred by the antigen. In contrast, Webster and Reynolds (37) stated that the agglutination-lysis titre following immunization with a dead antigen provided no measure of the degree of protection conferred. It has been observed by York (80) and Brown et al (78) that even when serum titres were not high following immunization adequate protection was afforded.

The effect of the challenge dose of live leptospirae upon the serum antibody titres of previously immunized animals has been commented upon by several researchers. York and Brueckner (75), Scheidy (86) and Kiesel and Dacres (88) observed no significant rise in the agglutination-lysis titre of immunized cattle following challenge of their immunity with live organisms. Morter et al (76) noted a transient rise to 1:10,000 in the agglutination-lysis titre in immunized swine following challenge of immunity.

All of the immunologic studies cited previously have been conducted with whole culture material. These investigations have also been concerned with methods of cultivation and inactivation of the organism to preserve antigenicity. Very few workers have attempted fractionation of the organisms and isolation of the antigenic components. The apparent lack of interest probably lies in the fact that yields of leptospirae from culture material are very small and the isolation of pure and unaltered components will be extremely difficult.

Some fractionation studies have been conducted with a view to the isolation of components which would be useful in serological tests. Schneider (89-93) published a series of papers describing the preparation of four fractions from leptospiral organisms. His procedure is summarized briefly as follows. The leptospirae were harvested by centrifugation, washed, resuspended in buffer to two per cent of the original volume and then disrupted by the addition of a solution of sodium desoxycholate. After shaking with a chloroform n-amyl alcohol solution, the mixture was separated by centrifugation into an upper aqueous phase, an interfacial cell protein phase and a lower chloroform phase. The dialysed aqueous phase constituted fraction 1. The interfacial layer was treated with tungstic acid and yielded a tungstic acid soluble phase, fraction 4, and a tungstic acid insoluble phase. Addition of 75 per cent alcohol to the latter, yielded an alcohol soluble phase, fraction 2 and an alcohol insoluble phase, fraction 3. The chloroform layer containing lipid was discarded. The procedure described yielded four relatively impure fractions all of which contained varying amounts of pentose polysaccharide. Fraction 3 was poor in

polysaccharide but contained most of the protein. All showed group reactivity in the complement-fixation test but fraction 3, the so-called protein fraction, was weak.

Rothstein et al (94-96) also prepared extracts of leptospirae and studied their properties. The cells were sedimented from culture by centrifugation and the sediment extracted with 70 per cent ethanol. After centrifugation, the supernatant fluid was saved and the sediment discarded. Following dialysis and pervaporation the material became opalescent. Centrifugation yieled a clear supernatant referred to as "precipitinogen" and a water insoluble sediment termed the "complement-fixation antigen". Rothstein postulated that leptospiral cells contained at least two antigens, a peripheral type-specific principle (P antigen) and a somatic genus-specific principle (S antigen). The S antigen was thought to be a lipopolysaccharide but the composition of the P antigen was not identified. It was concluded that the precipitinogen fraction contained both P and S antigens.

It will be apparent that very little is yet known about the antigenic structure of leptospirae.

LABORATORY METHODS FOR THE STUDY OF LEPTOSPIRAE

Microscopy

Leptospira are not visible by light microscopy because of their small diameter. They do not stain with aniline dyes but stain black in tissue sections by Levaditi's silver impregnation method and can be recognized by their morphology. Dark-field microscopy is most useful and living or dead organisms shine brightly against the dark background.

Wet mounts of culture, blood, urine, tissue fluids or ground tissue suspension may be examined by this method. Leptospirae must be distinguished from "pseudospirochetes" or artefacts such as erythrocyte extrusions and fragments of fibrin. A recently developed immunofluorescence technique (97-99) utilizes the dye fluorescein isothiocyanate. Immune globulins are conjugated with the fluorescent material. Preparations to be stained are then exposed to the labelled antibody which attaches specifically to the leptospirae. The organisms coated with labelled antibodies fluoresce brightly when the preparation is examined by dark-field microscopy utilizing an ultra-violet light source. None of the methods of microscopy permit more than tentative identification. However, in addition to revealing the morphology of the organism the fluorescent antibody technique utilizes a specific antigen-antibody reaction.

Serology

The more commonly accepted serological tests for the demonstration of leptospiral antibody are the complement-fixation test, the agglutination-lysis test and the plate-agglutination test. Of the three the agglutination-lysis test is the most widely used.

Many techniques based on complement-fixation have been described. The antigens used have been various preparations of culture supernatant liquor (79,100), chorio-allantoic fluid of infected chick embryos (101), cultures of organisms treated by heat (69, 102), chemicals (103, 104) or sonic vibration (105) and extracts of leptospiral cells (89-96, 102, 106). Complement-fixation antigens generally show a broad range of cross-reactivity with heterologous serotypes. Initial detection of antibodies is made about the 10th day of disease but may sometimes be made earlier. Antibodies are

usually not demonstrable by this method after three to four months (107).

Schuffner and Mochtar (108) are credited with the development of the agglutination-lysis (A L) or microscopic agglutination test. However, Noguchi (69) in 1920 described a simple test which was basically the same. The test utilized the fact that leptospirae agglutinate and appear to lyse in the presence of specific immune serum. One volume of live culture is mixed with an equal volume of each serum dilution to be tested and the tests are incubated. Drops of each dilution are examined for agglutination or lysis by dark-field microscopy. Studies by Lawrence (109) indicated that lysis is only apparent and that in the lower serum dilutions leptospirae have agglutinated more tightly and completely producing granular structures, the "lysis granules". The test is highly sensitive and serotype specific. Low titres may be detectible for years after infection (110). In a variation of the test which employs formalized antigen, agglutination occurs but lysis is not apparent.

A plate-agglutination test developed by Stoenner (111) utilizes a concentrated killed suspension of leptospirae as antigen. The serum titre is approximately 100 fold lower than that obtained with the agglutination-lysis test and cross reactions are broader.

Each of the three tests has its merits and disadvantages. The complement-fixation test being broadly reactive permits the use of a single antigen to detect recent infection due to various serotypes. It is a test that requires careful standardization. The plate test is a useful and simple screening test, but has the disadvantage that it does not cross react with all serotypes. The agglutination-lysis test is useful in

determining the serotype involved in an outbreak of leptospirosis but is cumbersome and requires the maintenance of a battery of live cultures for antigen.

Many other tests have been proposed in the literature for special purposes but have not received general acceptance. These include the hemolytic test (112), latex agglution test (113), capillary-tube test (114), milk agglutination test (115) and gel diffusion technique (116).

Isolation of Organisms

Animal inoculation has been most useful for the isolation of leptospira from tissues and fluids. The material is injected into the animal and at the height of temperature five or six days after inoculation heart blood is withdrawn and placed in culture media. The rapid invasiveness of leptospirae in the animal body may be utilized for the separation of leptospirae from contaminated cultures. The contaminated culture is injected intraperitoneally and five to ten minutes later heart blood is drawn and placed in culture media.

The most commonly used laboratory animals are guinea pigs and hamsters. Guinea pigs are perhaps more suitable since larger inocula may be given, the animals' temperatures may be taken and larger yields of sera are obtainable for tests of antibody development.

Leptospirae cannot be grown in the usual culture media. Special media are used consisting of a buffer salt base, pH 7.2, to which rabbit serum and haemoglobin have been added to supply a growth factor. Liquid media described by Korthof (117) and Stuart (118) are most commonly employed. Cultures are incubated at room temperature or in the incubator at 30°C.

Since leptospirae do not proliferate as rapidly as other bacteria the cultures may be overwhelmed and killed if the inoculum is contaminated. In addition, blood and tissue, particularly kidney tissue, contain inhibitary substances and it is therefore very important that small inocula be used.

Semi-solid media such as Fletcher's (119) are liquid media with the addition of one to two per cent agar. Semi-solid media are useful for the maintenance of stock cultures since the organisms grow slowly and remain viable for months in the dark at room temperature. It is claimed that virulence is also maintained longer in semi-solid medium.

Solid media have been described by Cox (120) but have not been found as useful as liquid or semi-solid media for the usual laboratory requirements. Solid media might prove useful for the isolation of single colonies and for the study of pure cultures.

EXPERIMENTAL STUDIES

The relative susceptibility of several species of laboratory animals to L. pomona infection has been investigated by Ringen and Okazaki (67) but calves and pigs were not included in their studies. The size of the experimental animal species has not been considered as a factor influencing the amount of the test dose. Such a consideration is generally reserved for non-replicating inocula such as toxins, drugs or antigens. However, leptospirae are extremely invasive and soon leave the site of inoculation to be carried throughout the body in the blood stream. Such rapid dilution of the inoculum suggested that body size might be of importance in the investigation of relative susceptibility.

References previously cited indicate that considerable study has been devoted to the stimulation of artificial immunity by the use of killed suspensions of leptospirae. The study of fractions of the leptospiral cells has been limited largely to their use as test antigens.

The results obtained in any study of susceptibility or immunity are influenced by the criteria used to evaluate them. The best measure of immunity is the response to challenge material of known virulence but this method is costly and extremely cumbersome, especially when conducted with large animals. In vitro tests have been substituted but some aspects of their mechanism of action and of their evaluation requires further study.

MATERIALS AND METHODS

Media and Cultures

Media

Stock cultures were maintained in Fletcher's (119) semi-solid medium which was prepared as follows. To 1610 ml. of sterile distilled water 240 ml. of sterile rabbit serum was added. The distilled water was sterilized in the autoclave at 15 pounds pressure for 30 minutes. The rabbit serum was passed through a Seitz filter using a D-8 steriflo pad. Melted meat extract agar was made by putting together 0.3 per cent beef extract, 0.5 per cent Difco proteosepeptone no. 2, 2.0 per cent agar and 150 mil. distilled water. The temperature was kept below 56°C. The melted meat extract agar was mixed with the diluted serum. The medium was dispensed in 5 ml. amounts in sterile screw cap tubes and inactivated by incubation at 56°C. for 60 minutes on two successive days.

For infective material and the production of antigens, the cultures were transferred from semi-solid medium to Korthof's liquid medium (117) which was prepared as follows:

Proteose peptone # (Difco)	0.8 gm.
NaCl	0.4 gm.
$NaH\ CO_3$	0.02 gm.
K Cl	0.04 gm.
$Ca\ Cl_2$	0.04 gm.
$KH_2\ PO_4$	0.24 gm.
$Na_2\ HPO_4$	0.70 gm.
H_2O	1 litre

The pH was 7.2 and required no adjustment. The mixture was brought to a boil for about one minute then filtered. To filter, a pulp made from #90 filter pads was placed over a #50 pad in a small Buchner funnel which was then sterilized in the autoclave before use. After filtration the base medium was dispensed in 10 ml. amounts in screw cap tubes and was sterilized by flowing steam for 30 minutes on each of three consecutive days. Prior to use 0.7 ml. sterile normal rabbit serum containing haemoglobin was added to each tube.

To obtain the serum-haemoglobin component a rabbit was bled by cardiac puncture and the blood was allowed to clot at 37°C. The serum was removed after centrifugation and inactivated in a water bath at 56°C for 30 minutes. Haemoglobin was prepared by adding an equal volume of distilled water to the clot and alternately freezing and thawing three or four times. A dry ice-alcohol bath was used to freeze the mixture.

The resulting solution was centrifuged to remove coarse particles and was added to the serum to a volume of ten per cent. The mixture was then sterilized by Seitz filtration through a D-8 sterilflo pad and was stored at 4°C until required.

Before use the media was checked for sterility and for its ability to support growth of leptospirae.

Cultures

Two cultures of L. pomona were selected. L. pomona (Wickard) was used for all experiments requiring live culture for infective material. The strain was selected because of its known pathogenicity. The original culture was obtained from the Veterinary Medical Research Institute, Iowa State College, Ames, Iowa. It had been maintained in semi-solid culture medium since that time. Routine transfers in this medium were made every two months. After primary incubation for two weeks at 30°C the stock cultures were checked by dark-field microscopy and those showing satisfactory growth were stored at room temperature in the dark. For experimental use two or three subcultures were made in liquid medium to ensure more uniform concentration of challenge material. When used the cultures were approximately seven to ten days old.

The strain of L. pomona selected for antigen preparation was obtained in 1956 from Dr. R.J. Avery of the Pacific Area Animal Pathology Laboratory in Vancouver, B.C. The strain was employed in all in vitro tests requiring either live or killed antigen material. It had been utilized for this purpose for the past five years and had been found to

be superior to other strains as test antigen. Cultures were maintained in liquid media and transfers were made according to requirements for antigen at maximum intervals of two weeks. During the present experiments live antigens were approximately seven to ten days old when used.

Estimation of density of cultures were made in two ways. For infective material bacterial cell counts were made with the Petroff - Hausser counting chamber. Before use the chamber, cover slip and red blood cell pipette were cleaned with distilled water, followed by alcohol and ether. Diluent was prepared by adding 3.2 ml. formalin to 38.6 ml. phosphate buffer pH 7.2. The pipette was filled to the 1.0 mark with live culture and then to the 101 mark with formalized buffer. A broad rubber band was placed over the ends of the pipette which was shaken vigorously for two minutes. Five drops were discarded. Then a small drop was placed on the counting chamber and allowed to spread across the chamber under the coverslip. Care was taken that none ran into the moats of the counting chamber. The chamber was placed in readiness on the stage of the microscope and allowed approximately ten minutes to settle. Immersion oil was placed on the dark-field condenser and counts were made using the 40 x objectives and 12.5 x occulars. All of the 400 small squares were counted. The focus was adjusted up and down in each field to scan the full depth of the chamber. Calculations were based upon the following formula.

$$\frac{\text{Number of organisms counted x dilution x number of small cubes in one ml.}}{\text{number of small squares counted}}$$

or

$$\frac{\text{Number of organisms counted x 100 x 20,000,000}}{400}$$

Cultures used for test antigens were selected from those showing satisfactory density as indicated by heavy growth with organisms almost touching each other. This was judged by dark-field examination of a drop of culture thinly spread between a clean microscope slide and a coverslip. Cultures were not used either for infective material or for antigen if motility was sluggish or if there was evidence of spontaneous agglutination.

Methods for Demonstration of Leptospirae

Darkfield Examination

Urine.- Two techniques were employed in the examination of urine. Upon collection 9 ml. of urine was placed in each of two tubes, one containing 1.0 ml. of phosphate buffer pH 7.3 and the other containing the same amount of buffer to which formalin had been added to a final concentration of ten per cent. A drop of the buffered urine was placed on a clean slide and covered with a coverslip. Examination was made using the dark-field condenser and 125 diameters magnification. The formalized sample was centrifuged at 2500 r.p.m. for 15 minutes in an International Centrifuge Model SBV, Size 1. A drop removed from the bottom of the tube with a Pasteur pipette was placed on a glass slide, covered with a coverslip and examined by dark-field microscopy.

Blood.- Nine ml. of fresh blood was mixed with 1.0 ml. of a one per cent solution of sodium oxalate in phosphate buffer pH 7.3. The cells were sedimented by centrifugation at 2500 r.p.m. for ten minutes. The plasma was examined by dark-field microscopy.

Culture Technique

Cultures of freshly-collected blood were made in Korthof's fluid medium and in Fletcher's semi-solid medium. Inoculum consisted of three drops of blood drawn at the height of temperature.

Cultures from tissues were made by grinding one gram of tissue in a Tenbroeck grinder with 10 ml. of Korthof's base medium as diluent. One ml. of this material was transferred to a tube of Korthof's medium and mixed. From this 1:10 dilution of tissue suspension, three drops were inoculated in semi-solid medium. Two additional dilutions, 1:100 and 1:1000, were also made in Korthof's medium

Animal Inoculation Technique

The presence of leptospirae in body fluids and tissues, especially when bacterial contamination was suspected, was made by the inoculation of guinea pigs. Blood collected at the height of temperature was defibrinated by shaking with glass beads. One ml. of the defibrinated material was injected subcutaneously into guinea pigs.

Fluorescent Antibody Technique

The techniques followed were basically those of Coons and Kaplan (97). To obtain hyperimmune serum a rabbit was injected intravenously three times at four day intervals with 10 ml. doses of live culture of L. pomona. Blood was collected two weeks following the last injection. The proteins were precipitated from the serum by 50 per cent saturation with ammonium sulphate. The ammonium sulphate was removed by dialysis against

physiologicalsaline. Protein content of the precipitate was determined by the micro Kjeldahl technique as described by Hawk and Bergeim (121). A sodium chloride-bicarbonate-acetone buffer mixture was added to make a final concentration of 10 mg. of globulin per ml. of solution. Commercially prepared fluorescein isothyocyanate powder was dissolved in acetone and this was added to the protein in amounts necessary to make a 1:20 concentration when added to the cold globulin solution. This was allowed to conjugate for 18 hours at 4°C with constant stirring. Excess dye was removed by dialysis against phosphate buffer (0.01 M. - pH 7.2). After filtration the conjugated stain was stored in the frozen state until used.

Urine smears were prepared from the sediment of formalized buffered samples. They were fixed with absolute ethanol for 15 seconds. When dry, the slides were flooded with the labelled antibody stain and allowed to react for one hour in a moist chamber at 37°C. They were then immersed in 0.85 per cent sodium chloride for ten minutes followed by immersion in distilled water for a further ten minutes. The slides were allowed to dry and were covered with a coverslip using a drop of glycerine containing ten per cent physiological saline as a mounting medium. The microscope was equipped with a pressure mercury vapour lamp OSRAM, HBO 200. Two BG 12 exciter filters were inserted to give a blue-violet light in the 5000-8000 A range. Blue barrier filters numbers OG 4 and GG 4 were placed in the extension tubes of the microscope. The dark-field condenser was used.

Tissue was examined in the same manner as the urine with the

exception that light touch impressions were made of the cut surface of the organ. Fixation was by means of four hours exposure to acetone at -15 to -20°C. Staining time was one hour.

Experimental Animals

All animals were examined and determined to be in good health before being accepted for experimental purposes. At the commencement of each experiment, animals were given identification tags, weights and temperatures were recorded and a blood sample was collected for an agglutination-lysis test. Antibiotics were not incorporated in any of the animal feeds.

Guinea pigs

Young female guinea pigs approximately two to three months old were obtained from a breeding colony on the premises.

Hamsters

Young hamsters of mixed sexes were employed when approximately four weeks old. The animals were also raised on the premises.

Rabbits

Young female rabbits approximately two months old were selected. These animals were raised on the premises.

Swine

Young Yorkshire pigs of mixed sexes were $2\frac{1}{2}$ to three months old when utilized. The animals were purchased from local farms but had been on the premises for over a month before being used.

Calves

Calves of mixed breeding and sex were obtained from local farms and from a herd maintained on the premises. All animals were young but ages varied somewhat in different experiments according to availability of animals and the purpose of the experiment.

Detection of Antibodies

Three tests were employed for the detection of circulating antibodies; the agglutination-lysis test, the complement-fixation test and the plate-agglutination test.

Agglutination-lysis Test

This test is based on the observation that a living leptospiral culture undergoes agglutination and lysis when in contact with serum containing homologous antibodies. The test antigen consisted of a seven to ten day-old culture grown in Korthof's medium. Before use the cultures were examined by dark-field microscopy to check the density and guard against the use of organisms exhibiting spontaneous agglutination.

The sera being tested were diluted with physiological saline to provide ten fold dilutions from 1:10 to 1: 1,000,000. These dilutions were pipetted in 0.1 ml. amounts into small serological tubes and 0.1 ml. of live antigen was added to each. The racks were shaken and the tests were allowed to incubate for four hours in a water bath at 37°C. Known positive and negative sera and saline antigen control tubes were also included. Readings were made by examining a drop of the serum-antigen mixture, thinly spread

between a clean slide and a cover slip, by dark-field illumination at a magnification of 125 diameters.

A serum was judged to be negative when the leptospirae remained uniformly spread throughout the field and of the same density in all serum dilutions and similar to the antigen control with saline. Reactions were graded as follows:-

4+ = complete disappearance of leptospirae or only one or two per field

3+ = almost complete disappearance or agglutination

2+ = about half the organisms not apparent or agglutinated

1+ = a few organisms agglutinated

Reactions of 2+ (50 per cent) were considered to be of sufficient magnitude to be classified as positive in that dilution.

Complement-fixation Test

The complement-fixation method followed closely the one described in the Standard Methods of the New York State Department of Health (122). The antigen used was a water insoluble fraction of an alcohol extract of L. pomona organisms as prepared by Rothstein and Hiatt (94) and will be described in more detail in a later section of this paper. The sera were tested in serial dilutions from 1:5 to 1:100. Three 50 per cent haemolytic units of complement were used. All reagents of the tests were added in 0.1 ml. amounts. The period of preliminary incubation was 18 hours at approximately 9°C., that of secondary incubation after the addition of the sensitized sheep red cells, was 30 min. at 37°C. The serum titre was recorded as the highest dilution with which 50 per cent haemolysis or less

was obtained in the presence of antigen.

Plate-agglutination Test

The antigen was prepared according to the method of Stoenner (111) modified as follows. The leptospirae were grown in Korthof's medium distributed in 100 ml. amounts in eightoz. screw cap bottles. The incoulated medium was incubated for seven days at 30°C, and the cultures showing good growth were killed by the addition of 1.0 per cent formalin. The following day, the organisms were separated by centrifugation for 10 min. at 10,000 r.p.m. then resuspended in a borax-boric acid buffered solution, Buffer A, containing ten per cent formalin in physiological saline. The suspension was adjusted to a concentration seven times McFarland's nephalometer tube no. 1. After standing for four days at 9°C the antigen was centrifuged and resuspended in the same volume of Giemsa-buffer solution. This was prepared by adding 2 ml. of concentrated Giemsa stain to each 8 ml. of Buffer B and centrifuging before incorporating the organisms. Any precipitated or clumped organisms that formed on standing were resuspended by repeated aspiration with a 10 ml. syringe equipped with a #20 gauge needle. The clumps that were not resuspended by this procedure were discarded.

To perform the test, 0.03 ml. of stained antigen was mixed with 0.01 ml. of undiluted serum and serum dilutions of 1:10, 1:40, 1:160 and 1:640 on a glass plate and incubated over a specially-designed box equipped with indirect lighting. The plate was tilted a few times then allowed to stand on the incubation box for two minutes. The plate was again tilted several times and the reactions were recorded.

RESULTS

Protocols for the experiments and the results obtained will be combined for greater coherence. The results of the three parts of the project will be described separately as follows:

Part I. The relative susceptibility of various species of animals to L. pomona infection.

Part II. The immunological reactivity of several species of animals to killed suspensions of L. pomona and fractions thereof.

Part III. The investigation of some methods presently used to determine infection and immunity.

Part I. Relative Susceptibility

In the course of determining the relative susceptibility of several animals species to L. pomona infection we first determined the infectivity of the organism for guinea pigs. Multiples of the guinea-pig infective dose were then injected according to weight in an attempt to infect other animal species.

Susceptibility of Guinea Pigs

Experiment 1.- Forty guinea pigs averaging 496 gm. were used in this experiment. These were divided into ten groups of four animals per group by stratified randomization using four strata according to weight.

An eight day culture of L. pomona (Wickard) was examined by dark-field microscopy to assure good growth, active motility and absence of spontaneous clumping. Three cell counts were made which resulted in

figures of 250,000,000; 260,000,000 and 240,000,000 per ml. respectively. The average count of 250,000,000 leptospirae per ml. was used for calculating dilutions. Dilutions were prepared using Korthof's base medium as diluent. A separate pipette was employed to mix and transfer each dilution. One ml. of culture was mixed with 11.5 ml. of diluent to make a 1:12.5 dilution or 20,000,000 leptospirae per ml. Two further ten-fold dilutions resulted in a 1:1250 suspension or 200,000 leptospirae per ml. Thefirst inoculum consisted of 0.5 ml. of this dilution or 100,000 leptospirae. Succeeding two-fold dilutions were made by adding 3 ml. of the preceeding suspension to 3 ml. of diluent. The 10th and final suspension was a 1:640,000 dilution which provided 195 leptospirae per 0.5 ml. inoculum. Group numbers and inocula used were as follows:

Group	Dilution	No. of leptospirae
1	1:640,000	195
2	1:320,000	390
3	1:160,000	781
4	1: 80,000	1,563
5	1: 40,000	3,125
6	1: 20,000	6,250
7	1: 10,000	12,500
8	1: 5,000	25,000
9	1: 2,500	50,000
10	1: 1,250	100,000

Each animal was injected subcutaneously in the left side just behind the foreleg. Temperatures were recorded daily for 14 days after exposure. Blood was collected from all animals for the agglutination-lysis test on the 9th, 14th and 60th post-inoculation days. Cultures in Korthof's medium were made of heart blood drawn on the 9th day from the 12 animals which received the smallest numbers of organisms. Examination of these cultures 17 days later revealed leptospirae in all of them.

Table I gives the temperature record in detail since it revealed several interesting findings. Temperatures of 104°F and above were considered indicative of infection. All guinea pigs showed a temperature response which occurred between the 5th and 13th post-inoculation days. The animals which received the smaller numbers of leptospirae took up to three days longer to develop infection than those which received the larger numbers. Peak temperatures were as high in the two low dose groups as in the two high dose groups but fever was not of as long duration.

Table II reveals that all animals responded serologically. The agglutination-lysis titres after 14 and 60 days were essentially the same in all groups. The tests on the first six groups of guinea pigs performed on the 9th day after inoculation gave negative results. By this time the last four groups of guinea pigs which had received progressively larger numbers of leptospirae, had developed agglutination-lysis titres which increased proportionately with the numbers of leptospirae administered. From this it was evident that the animals which had received the larger numbers of leptospirae developed infection sooner after inoculation. Animals which showed borderline temperatures of approximately 104°F for a single day, as for example guinea pigs 233 and 238 developed titres similar to those showing a high temperature response.

Table III summarizes the relationship between the temperature response and the serological titres at the various dose levels. The severity of infection as indicated by the temperature response did not increase with the size of the infective dose. On the contrary, the animals

TABLE I

TEMPERATURE CHART OF GUINEA PIGS INJECTED WITH VARYING NUMBERS OF LEPTOSPIRAE

Group number	Leptospirae inoculated	Animal number	Daily temperature response[a]								
			5	6	7	8	9	10	11	12	13
1	195	228	102.7	102.6	103.6	105.4	104.2	102.5	103.4	102.6	102
		235	102.6	102.6	102.8	105	105.5	104.4	102.8	102.6	102.4
		409	102.7	102.6	102.6	103.8	105.6	103.8	103.3	102.8	102.2
		411	102.2	101.8	101.9	102.2	105.2	105.1	103.5	102.9	102.1
2	390	226	102.5	103.1	102.9	104.4	103.6	104.3	102.6	103.7	102.4
		232	102.5	102.8	102.9	102.6	103.8	105.1	103.6	102.9	102.4
		239	102.9	102.8	103.2	103	103.8	105.2	102.8	102.8	102.4
		408	102.3	102.6	102.8	103	104.2	104.3	106.4	102.9	102.2
3	781	240	102.8	102.8	104.2	106	104.6	103.1	104.4	103.4	102.6
		245	102.7	103	103	105.4	105.9	104.5	104.2	103.6	102.5
		249	102.3	102.5	102	104.2	104.7	104.8	103.6	103.5	102.8
		412	102.6	103	103.7	103.2	105.3	104.4	103.2	104.7	102.6
4	1563	247	103	103.2	105.6	105.6	103.8	104.4	103.8	102.2	103.3
		404	102.6	102.8	104	104	105.7	104.4	102.5	102.6	102.6
		406	102.3	103	104.8	105	104	103.5	103.5	102.6	102.5
		425	102.8	103.2	103.3	104.9	105.6	106.6	105	103.7	104.8
5	3125	423	102.6	103	103.2	105.3	104	104.6	103.7	103	102.2
		403	102.6	103	105.1	105.2	104.5	103.4	103.8	102.7	102.4
		413	102.8	103	105.7	105.1	106	100.5	104.4	103.6	103.3
		419	102.4	104.4	104.2	104.8	103.5	103.2	102.8	102.6	102

(continued)

TABLE I.- TEMPERATURE CHART OF GUINEA PIGS INJECTED WITH VARYING NUMBERS OF LEPTOSPIRAE (continued)

Group number	Leptospirae inoculated	Animal number	Daily temperature response[a] 5	6	7	8	9	10	11	12	13
6	6250	230	102.4	103	105.7	105.4	104.2	103.4	103	102.2	102.4
		216	102.5	103	104.6	105.1	104.6	104	103.6	102.8	102.2
		402	102.9	103.2	106	105	104.2	103.6	104.4	102.2	102
		410	102.7	104.4	105.6	103.5	102.8	104	102.5	103	102.7
7	12,500	229	102.8	103.8	104.3	105.5	104.2	105.4	102.6	102.1	102.2
		236	102.8	103.5	104.4	103	103	103	102.3	103	102.8
		244	102.5	103.7	105	105.2	104	103.8	102.4	101.6	102
		415	102.6	103.4	104.6	103.3	103	102.8	102.5	102.6	102.7
8	25,000	416	102.5	102.8	104.2	104.7	102.8	102.8	102.5	102.2	102.5
		420	103	104.2	104.4	104.8	103.5	103.8	102.6	102.5	102.8
		421	103.4	104.5	104.8	104.6	103.4	103.8	**102.4**	**102.2**	**101.9**
		422	103.2	104.6	105.1	103	102.6	102.7	102.6	102.4	102.5
9	50,000	233	103	104	102.7	103	103	102.2	102.8	102.9	102.9
		234	103.1	105.2	105.2	103	103.7	104	102.5	103.2	102.8
		238	103.2	104	102.4	103.2	103.5	102.5	102.1	102.4	102.2
		424	103	105.4	105.2	104.5	103.2	104.4	103.2	102.6	102.6
10	100,000	237	104.3	104	104.7	103.8	104	103.5	103.4	102.6	102.5
		417	102.9	105.3	104.2	103.4	103.5	103.8	102.2	102.4	103.2
		418	104.3	104	102.9	102.8	102.9	102	102.3	102.5	103.1
		423	104.4	104.7	103.9	104	103	101.6	102.6	103.1	103

[a]Temperatures for days 1-4 and 14 were within the normal range.

TABLE II

RESULTS OF AGGLUTINATION-LYSIS (AL) TESTS OF SERA FROM GUINEA PIGS INJECTED WITH VARYING NUMBERS OF LEPTOSPIRAE

Group no.	Animal no.	AL titre[a] 9th day	14th day	60th day	Group no.	Animal no.	AL titre[a] 9th day	14th day	60th day
1 (195)	228	-	1,000	1,000	6 (6,250)	230	-	1,000	1,000
	235	-	1,000	1,000		246	-	1,000	1,000
	409	-	1,000	1,000		402	-	1,000	1,000
	411	-	1,000	100		410	tr	1,000	1,000
2 (390)	226	-	1,000	1,000	7 (12,500)	229	-	10,000	1,000
	232	-	1,000	1,000		236	10	1,000	1,000
	239	-	1,000	1,000		244	-	1,000	1,000
	408	-	1,000	1,000		415	10	10,000	1,000
3 (781)	240	-	1,000	1,000	8 (25,000)	416	-	1,000	1,000
	245	-	1,000	DIED		420	10	1,000	1,000
	249	-	1,000	1,000		421	tr	1,000	1,000
	412	-	1,000	1,000		422	10	10,000	1,000
4 (1563)	247	-	10,000	1,000	9 (50,000)	233	100	10,000	1,000
	404	-	1,000	1,000		234	10	1,000	100
	406	-	1,000	1,000		238	100	1,000	100
	425	-	1,000	1,000		424	tr	1,000	1,000
5 (3125)	423	-	10,000	1,000	10 (100,000)	237	1,000	10,000	1,000
	403	-	1,000	1,000		417	10	1,000	1,000
	413	tr[b]	10,000	1,000		418	1,000	1,000	1,000
	419	tr[b]	10,000	1,000		423	1,000	10,000	1,000

[a]Agglutination-lysis titres shown are the reciprocal of the highest serum dilution giving a postive reaction. Tests were made on the post-inoculation days shown.

[b]Trace.

TABLE III

THE RELATIONSHIP BETWEEN SIZE OF INOCULUM, TEMPERATURE RESPONSE AND AGGLUTINATION-LYSIS TITRE IN GROUPS OF GUINEA PIGS INOCULATED WITH VARIOUS NUMBERS OF *L. POMONA*

Group number	Leptospirae per inoculum	Average peak temperature	Average time (days) to peak temp.	Average AL titres[b]		
				9th day	14th day	60th day
1[a]	195	105.4	8.75	-	1,000	775
2[a]	390	105.3	9.75	-	1,000	1,000
3[a]	781	105.5	9	-	1,000	1,000
4	1,563	105.7	8.625	-	3,250	1,000
5	3,125	105.3	8.25	tr.	5,500	1,000
6	6,250	105.6	7.25	tr.	1,000	1,000
7	12,500	104.9	7.5	5	5,500	1,000
8	25,000	104.9	7.5	5	3,250	1,000
9	50,000	104.7	6.375	50	3,250	550
10	100,000	104.8	6	750	5,500	1,000

[a]Cultures made from heart blood of all animals in these three groups were positive.
[b]Titres are expressed as the reciprocal of the highest serum dilution giving a positive reaction.

which received the higher dose levels tended to have average peak temperatures of 104.7 to 104.9°F while those which received the lower dose levels showed average peak temperature of 105.3 to 105.5°F. However the average time required to reach peak temperature was different: approximately six days after the higher dose levels and approximately nine days after the lower dose levels. Nevertheless, the ultimate 60 days agglutination-lysis antibody titre was unaffected by the size of the initial infective dose.

The temperature response developing in all guinea pigs, 104°F to 106.6°F, the agglutination-lysis titres ranging from 1:1000 to 1:10,000 and the positive cultures of heart blood indicated that all 40 animals had developed leptospirosis.

Since the minimum infective dose of L. pomona for guinea pigs was not attained in this trial, another was made using fewer organisms.

Experiment 2.- Twenty guinea pigs averaging 388 gms. were used in the second experiment. These were divided into five groups of four animals per group by stratified randomization according to weight.

A well grown 11-day culture of L. pomona (Wickard) was selected for use. Three cell counts were made which resulted in figures of 235,000,000, 240,000,000 and 235,000,000 per ml. respectively. The average count of 237,000,000 leptospirae per ml. was used for calculating dilutions. One ml. of culture was mixed with 10.85 ml. diluent to make a 1:11.85 dilution or 20,000,000 leptospirae per ml. Four ten-fold dilutions reduced the number to 2,000 per ml. A further 2:3 dilution was made to produce 800 leptospirae per ml. From this, five two-fold

dilutions were made to produce suspensions of from 200 down to approximately 12 or 13 leptospirae per 0.5 ml. inoculum. Group numbers and inocula used were as follows:

Group	Dilution	Inoculum
1	1:9,480,000	13
2	1:4,740,000	25
3	1:2,370,000	50
4	1:1,185,000	100
5	1: 592,500	200

Each animal was injected subcutaneously in the left side just behind the foreleg. Temperatures were taken daily for 16 days after exposure. Blood was collected from all animals on the 8th post-inoculation day for culture and again on the 15th, 30th and 60th days for the agglutination-lysis test.

Table IV reveals that all guinea pigs showed some pyrexia between the 6th and 14th post-inoculation days. Table V indicates that on the basis of the agglutination-lysis titres of their sera all animals except one developed leptospirosis. The single exception, No. 775, showed a low unexplained increase in temperature. Leptospirae were recovered by cultural methods from the heart blood of 15 of the 20 animals.

The end point of infectivity was not attained even at the high dilution corresponding to 13 leptospirae in the0.5 ml. inoculum. The erratic temperature response, particularly in the low dose levels, suggested that the experiment had exceeded the confidence range with respect to the accuracy of the dilutions. Since the group of guinea pigs which received 50 leptospirae in the inoculum was the last showing a regular response to inoculation of infective material, this number was selected for use in

TABLE IV

TEMPERATURE CHART OF THE SECOND SERIES OF GUINEA PIGS INOCULATED WITH VARYING NUMBERS OF LEPTOSPIRAE

Group number	Leptospira inoculated	Animal number	Post Inoculation Temperatures[a]						
			8	9	10	11	12	13	14
1	13	759	103	104.4	105.2	104.5	104.4	103.8	103
		764	103.6	103.8	105.6	105.4	103.2	102.8	103
		772	102.5	105.8	106	104.5	103.2	103.2	102.6
		775	103.6	103.7	103.6	104.2	103.8	103	103
2	25	755	103.6	106	105.4	103	103	102.7	102.4
		761	103	103.2	102.8	102.2	104	104.6	106.2
		769	102.8	102.6	102.6	103.5	105	104.2	103
		757	102.6	102.8	105.6	104.8	105.4	103	103.2
3	50	758	105.2	105.4	102.6	103.8	102.5	102.5	102.4
		771	105	105.8	105.4	103	104	102.3	102.2
		752	103.6	105.8	105.3	103.4	104	102.4	102.6
		760	103.3	105	105.4	105	104.2	104	103.8
4	100	762	103	105.5	105.8	103.4	104.2	103.4	102.2
		768	103.2	103.2	104.4	105.4	103.5	103.2	103
		763	103	103.4	105.4	105.0	103	103.2	103.4
		753	103	103.3	103.6	105.4	104.4	103.6	104.2
5	200	751	103	105.0	105.6	103	103.4	102.8	102.8
		765	105.3	105.6	104.4	103.8	102.5	102.2	102.8
		754	103.6	105	106	102.2	103	102	102.3
		767	104.8	105	104	103	102.8	102.8	102.6

[a]Temperatures during days 1 - 7 and 15 and 16 were within the normal range with the exceptions of animal 768 which showed a temperature of 104.1°F on day 6 and animal 775 which had a temperature of 104° on day 7.

the study of the relative susceptibility of the other animal species.

Relative Susceptibility of Various Species

From the foregoing experiment the guinea pig infective dose was estimated to be 50 leptospirae for a 388 gm. animal. Multiples of this dose were inoculated subcutaneously into guinea pigs, hamsters, rabbits, calves and pigs according to their body weight. The susceptibility to infection was based upon temperature response, the appearance of antibodies and the recovery of the organism.

Guinea Pigs.- Twelve animals averaging 556 gm. were divided at random into three groups of four guinea pigs per group. Each group was inoculated according to weight with 1/10, one and ten guinea pig infective doses respectively, that is with seven, 70 and 700 organisms.

Hamsters.- Twelve animals averaging 78 gm. were divided at random into three groups of four animals. Each group was inoculated according to weight with 1/10, one and ten guinea pig infective doses respectively. Inocula contained one, ten and 100 leptospirae.

Rabbits.- Four animals averaging 988 gm. were used in a single group which received one multiple of the guinea pig dose or 125 leptospirae.

Calves.- Four animals averaging 93,327 kg. were used in a single group which received one multiple of the guinea pig infective dose according to weight or 12,000 leptospirae

Swine.- Twenty eight pigs averaging 33.307 kg. were divided by stratified randomization into four groups of seven animals each. A group of pigs received 1/10, one, ten and 100 guinea pig infective doses respectively. Inocula contained 420, 4,200, 42,000 and 420,000 leptospirae.

TABLE V

RESULTS OF BLOOD CULTURE STUDIES AND SERUM AGGLUTINATION-LYSIS TESTS ON THE SECOND SERIES OF GUINEA PIGS INOCULATED WITH VARIOUS DOSE LEVELS OF LEPTOSPIRAE

Group number	Animal number	Blood Culture	Agglutination-lysis titres[a]		
			15th day	30th day	60th day
1	759	+	1,000	1,000	1,000
	764	+	1,000	1,000	1,000
	772	+	1,000	1,000	1,000
	775	-	-	-	-
2	755	+	1,000	1,000	1,000
	761	-	10	1,000	1,000
	769	-	10	1,000	1,000
	757	-	100	1,000	1,000
3	758	+	1,000	10,000	1,000
	771	+	1,000	1,000	100
	752	+	1,000	10,000	1,000
	760	+	100	10,000	1,000
4	762	+	1,000	1,000	1,000
	768	-	1,000	1,000	1,000
	763	+	100	1,000	1,000
	753	+	1,000	1,000	1,000
5	751	+	1,000	1,000	1,000
	765	+	10,000	1,000	1,000
	754	+	1,000	1,000	1,000
	767	+	10,000	1,000	1,000

[a]Titres are expressed as the reciprocal of the highest serum dilution giving a positive reaction.

The average of the cell counts of the culture of L. pomona used to make the inocula was 180,000,000 leptospirae per ml. The dilution scheme used to arrive at the appropriate inocula for the various groups and species is shown in detail. The inocula for the guinea pigs, hamsters and rabbits were prepared in 0.5 ml. volumes. The calves and swine received inocula in 2 ml. volumes.

Tube no.	Dilution	Leptospirae per ml.	Inoculum
1	culture	180,000,000	
2	1 ml. tube 1+9 ml. diluent	18,000,000	
3	1 ml. tube 2+9 ml. "	1,800,000	
4	5 ml. tube 3+5 .71 ml. "	840,000	
5	6 ml. tube 4+6 ml. "	420,000	
			(Swine)
6	10 ml. tube 5+10 ml. "	210,000	420,000 per 2 ml.
7	2 ml. tube 6+18 ml. "	21,000	42,000 per 2 ml.
8	2 ml. tube 7+18 ml. "	2,100	4,200 per 2 ml.
9	2 ml. tube 8+18 ml. "	210	420 per 2 ml.
10	1 ml. tube 5+9 ml. "	42,000	
11	3.6 ml. tube 10+9 ml. "	12,000	
			(Calves)
12	5ml. tube 11+5 ml. "	6,000	12,000 per 2 ml.
13	2 ml. tube 10+8 ml. "	8,400	
			(Guinea pigs)
14	2 ml. tube 13+10 ml. "	1,400	700 per 0.5 ml.
15	1 ml. tube 14+9 ml. "	140	70 per 0.5 ml.
16	1 ml. tube 15+9 ml. "	14	7 per 0.5 ml.
			(Rabbits)
17	1 ml. tube 14+4.6 ml. "	250	125 per 0.5 ml.
			(Hamsters)
18	1 ml. tube 14+6 ml. "	200	100 per 0.5 ml.
19	1 ml. tube 18+9 ml. "	20	10 per 0.5 ml.
20	1 ml. tube 19+9 ml. "	2	1 per 0.5 ml.

The guinea pigs, hamsters and rabbits were injected subcutaneously in the right side just behind the foreleg. The calves were injected subcutaneously in the neck and the swine in the pectoral region. Temperatures were recorded daily for 15 days in the guinea pigs and rabbits, for 17 days

in the calves and for 21 days in the swine. Temperature determinations were not attempted in the hamsters. Blood was collected for culture from all animals which showed a temperature response. In the case of the hamsters, cultures were made of kidney tissue on the 21st post-inoculation day. Blood was collected at intervals for the agglutination-lysis test as indicated in Tables VI-X.

Table VI shows that three guinea pigs of four in group two became infected. This group had been inoculated with one guinea pig infective dose of 70 leptospirae. The same result was obtained in group 3 which had received ten times this number of leptospirae while two animals of group 1 became infected after receiving 1/10th the dose. While the remarkable infectivity of L. pomona for guinea pigs has been demonstrated, no sharp line of demarkation can be shown between an infective dose and a non-infective one.

Results of the tests in hamsters are shown in Table VII. Group 2 which received one guinea pig infective dose in proportion to weight, or ten leptospirae, had three infected animals among the four in the group. When ten times this dose was given, two animals became infected but none became infected when 1/10 the dose was given. On a weight basis the results obtained in the hamsters appear comparable to those found in the guinea pigs.

Rabbits can be infected with one guinea-pig infective dose relative to weight (125 leptospirae) as shown in Table VIII. However, only one of four rabbits in the group developed infection. This animal showed a high temperature response and agglutination-lysis antibody titre.

TABLE VI

TEMPERATURE RECORD AND RESULTS OF BLOOD CULTURE AND AGGLUTINATION-LYSIS TESTS IN GUINEA PIGS EXPOSED TO VARIOUS NUMBERS OF LEPTOSPIRAE

Group number	Guinea pig	Daily temperatures[b] 9	10	11	12	13	Blood cultures	AL titres 15 days[c]
1 (7 organisms)	815	105.2[a]	105.8	103.7	103.2	103.6	+	10,000
	808	104.4[a]	105.8	104.2	102.6	102	+	1,000
	821	102.6	102.5	103	102.6	102.7	ND	-
	810	102.8	102.8	102.4	102.7	102.5	ND	-
2 (70 organisms)	819	103.2	104.4[a]	104.4	103	103.1	+	1,000
	823	103	102.8	102.6	102.2	102.5	ND	-
	812	105[a]	105.9	104.4	103.5	103.5	+	1,000
	807	104.8[a]	104.6	102.5	103.5	102.4	+	10,000
3 (700 organisms)	801	103.8	105.2[a]	106	103.4	104.1	+	1,000
	818	103	103	104.4[a]	105.5	104.8	+	1,000
	806	103.2	103.2	105.4[a]	104.6	103.6	+	1,000
	825	103.3	103	102.2	103.2	103.4	ND	-

[a]Cultures of heart blood made in Korthof's medium.
[b]Temperatures for days one - eight and 14-15 were within the normal range.
[c]Titres are expressed as the reciprocal of the highest serum dilution giving a position reaction.
ND - not done.

TABLE VII

RESULTS OF SEROLOGICAL TESTS AND CULTURE OF KIDNEY TISSUE IN HAMSTERS FOLLOWING EXPOSURE TO VARIOUS NUMBERS OF LEPTOSPIRAE

Group number	Hamster number	Agglutination-lysis titre[a] 21st day	Kidney culture 21st day
1 (1 leptospira)	1	-	-
	2	-	-
	3	-	-
	4	-	-
2 (10 leptospirae)	5	-	-
	6	10	+
	7	10	+
	8	10	+
3 (100 leptospirae)	9	10	+
	10	-	-
	11	-	-
	12	10	-

[a]Titres are expressed as the reciprocal of the highest serum dilution giving a positive reaction.

TABLE VIII

TEMPERATURE RESPONSE AND RESULTS OF BLOOD CULTURE AND SEROLOGICAL TESTS IN RABBITS EXPOSED TO ONE GUINEA PIG INFECTIVE DOSE OF LEPTOSPIRAE

Rabbit identification	Temperature response[a]	Blood culture[a]	Agglutination-lysis titre[b]	
			14th day	28th day
Grey	-	-	-	-
Black	-	-	-	-
Brown	+	+	100,000	10,000
Spot	-	-	-	-

[a] 9th post-inoculation day.
[b] Titres are expressed as the reciprocal of the highest serum dilution giving a positive reaction.

Calves were shown to be as susceptible as guinea pigs to leptospiral infection when the infective dose was calculated relative to body weight. As indicated in Table IX, three of four calves developed the disease when injected with 12,000 organisms.

Swine were found to be very resistant to leptospiral infection. As summarized in Table X none of the animals could be shown to be infected even when 100 times the guinea pig dose was administered. Seven animals which were injected with the relatively large number of 420,000 leptospirae failed to become infected as compared with the calves in which 12,000 organisms were capable of producing disease.

These results indicate that when the natural resistance of young animals of various species is challenged on a body weight basis guinea pigs, hamsters, rabbits and calves are highly susceptible to infection. Swine appear to be much more resistant.

TABLE IX

TEMPERATURE RESPONSE AND RESULTS OF CULTURAL AND SEROLOGICAL TESTS IN CALVES EXPOSED TO ONE GUINEA PIG INFECTIVE DOSE OF L.POMONA

Calf number	Temperature response[a]	Blood culture	Kidney culture	Agglutination-lysis titre[b]		
				14th day	21st day	28th day
506103	-	-	-	-	-	-
506104	±	-	+	100	10,000	1,000
506107	+	-	+	100	100	100
506108	+	+	-	100	1,000	1,000

[a]Post-incoulation days 7 to 9.
[b]Titres are expressed as the reciprocal of the highest serum dilution giving a positive reaction.

TABLE X

TEMPERATURE AND SEROLOGICAL RESPONSE OF 28 SWINE INOCULATED WITH SEVERAL DOSE LEVELS OF LEPTOSPIRAE

Group number[a]	Leptospirae inoculated	Temperature response	Agglutination-lysis titre		
			14th day	21st day	28th day
1	420	-	-	-	-
2	4,200[b]	-	-	-	-
3	42,000	-	-	-	-
4	420,000	-	-	-	-

[a] Seven pigs per group.
[b] Multiple of one guinea pig infective dose proportional to weight.

PART II

Immunological Reactivity

The object of these experiments was to study the immune response of guinea pigs and calves to killed suspensions of L. pomona and fractions thereof.

Reactivity to Whole Killed Suspension

Seven killed suspensions of L. pomona were used. These had been prepared for immunological purposes by different biological laboratories. Characteristics of these antigens are shown in Table XI.

Guinea Pigs.- It was thought advisable to determine the post-immunization serological titres of guinea pigs in this experiment for comparison with those obtained following administration of infective material. However, the collection of a relatively large quantity of blood by cardiac puncture was considered to be too severe a stress to impose upon the animals just previous to the administration of the challenge material. One series of eight groups of guinea pigs, identified by the letter "A", were employed to determine post-immunization serological titres. The second series, identified by the letter "B" were not bled following immunization but were injected with challenge material.

Seventy-two guinea pigs averaging 300 gm., were divided at random into two series of eight groups per series. Series "A" contained four guinea pigs per group while series "B" contained five per group. One group from each series was inoculated with one of the seven killed antigens. The remaining group of each series was not inoculated and retained

TABLE XI

CHARACTERISTICS OF WHOLE CULTURE ANTIGENS OBTAINED FROM SEVERAL BIOLOGICAL LABORATORIES

Antigen number	Strain of *L. pomona*	Density[a]	Adjuvant	Inactivated by
1	Baker T262	11	None	formalin
2	"	25	$Al(OH)_3$	formalin
3	"	102	$Al(OH)_3$ 20%	thimersol
4	"	13	None	formalin
5	S91 and King B	98	$Al(OH)_3$ 10%	thimersol
6	Baker T262	25	$Al(OH)_3$ 5%	formalin
7	"	45	$Al(OH)_3$ 2%	formalin

[a]Determined with the Klett-Summerson photoelectric colorimeter using McFarland's nephalometer tube number 1 as the standard.

for control purposes. The antigens were diluted 1:4 with physiological saline and 1.0 ml. inoculum was administered subcutaneously in the left side behind the foreleg. Two weeks later blood was collected by cardiac puncture from all animals in series "A". After the same interval the immunity of all animals in series "B" was challenged by the subcutaneous inoculation of 100 minimum infective doses (m.i.d.) of leptospirae in defibrinated guinea pig blood. To determine the m.i.d. of this material two titrations were made. Blood was collected at height of temperature from two guinea pigs in the acute stage of leptospirosis. Ten-fold dilutions of the defibrinated blood from undiluted to 1:100,000 were made in physiological saline using a fresh pipette to mix and transfer each dilution. In both titrations one guinea pig was inoculated subcutaneously for each dilution of infective material. A temperature rise to 104°F. or above was considered indicative of infection. Table XII reveals the results of both titrations which showed the m.i.d. to be the 1:1000 dilution of pooled guinea pig blood.

Following challenge of their immunity with 100 m.i.d. of challenge material temperatures were recorded daily for 14 days. Temperatures of 104°F. and above or agglutination-lysis antibody titres of 1:1000 or above were considered indicative of infection. Blood was collected three weeks after challenge for the agglutination-lysis test.

Table XIII summarizes the serological results obtained in the agglutination-lysis test 14 days following immunization of guinea pigs in series "A". It will be noted that those animals that had received antigen 1 failed to show any trace of reaction even in the 1:10 serum dilution.

TABLE XII

RESULTS OF TWO TITRATIONS FOR INFECTIVITY OF GUINEA PIG BLOOD COLLECTED AT HEIGHT OF TEMPERATURE RESPONSE TO LEPTOSPIROSIS

Titration 1			Titration 2		
Animal number	Inoculum[a]	Temperature response	Animal number	Inoculum[b]	Temperature response
465	undilute	+	166	undilute	+
468	1:10	+	173	1:10	+
469	1:100	-	168	1:100	+
464	1:1000	+	164	1:1000	+
167	1:10,000	-	162	1:10,000	-
163	1:100,000	-	157	1:100,000	-

[a] 1.0 ml. amounts of pooled defibrinated blood collected from infected guinea pigs 463 and 467 and diluted as indicated.

[b] Pooled defibrinated blood collected from infected guinea pigs 153 and 154.

TABLE XIII

REACTIONS OBTAINED IN THE AGGLUTINATION-LYSIS TESTS OF GUINEA PIG SERA COLLECTED 14 DAYS AFTER IMMUNIZATION

Group number	Animal no.	Serum dilution 1/10	1/100	1/1000	A.L. Titre[a]	Group number	Animal no.	Serum dilution 1/10	1/100	1/1000	A.L. Titre[a]
1A	237	-	-	-	-	5A	233	1	-	-	-
Antigen	227	-	-	-	-	Antigen	241	-	-	-	-
1	235	-	-	-	-	5	240	1	-	-	-
	226	-	-	-	-		236	-	-	-	-
2A	249	2	-	-	10	6A	247	1	-	-	-
Antigen	242	4	3	1	100	Antigen	246	3	2	-	100
2	232	3	1	-	10	6	231	1	-	-	-
	244	3	2	-	100		234	2	1	-	10
3A	245	2	-	-	10	7A	230	1	-	-	-
Antigen	248	1	-	-	-	Antigen	274	1	-	-	-
3	239	1	-	-	-	7	256	1	-	-	-
	250	2	1	-	10		260	2	1	-	10
4A	243	2	1	-	10	8A	262	-	-	-	-
Antigen	238	-	-	-	-	Control	270	-	-	-	-
4	228	-	-	-	-		252	-	-	-	-
	229	3	1	-	10		275	-	-	-	-

[a]Titre is expressed as the reciprocal of the highest serum dilution giving a positive reaction.

All guinea pigs that were injected with antigen 2 developed positive serological reactions ranging from the 1:10 to 1:100 serum dilutions. The remaining five antigens stimulated serological responses of intermediate degree. On the basis of the serum titres obtained in the agglutination-lysis test it was concluded that antigen 1 gave the poorest and antigen 2 the best antigenic response.

Table XIV presents the results obtained in animals of series "B" which received infective material following immunization with the various antigen. It will be noted that of the five animals injected with antigen 2, four resisted infection. Three animals resisted infection in each of the groups injected with antigens 3 and 6, and two animals were resistant in each group injected with antigens 1 and 7. One animal each in groups 4B and 5B and in the control group resisted infection. Animals 973, 976 and 994 of groups 3B, 4B and 7B respectively, showed an increased antibody response without a corresponding temperature rise. It was also noted that three animals which became infected in group 1B showed a temperature response on the fourth post-challenge day as compared with similar animals in other immunized groups which did not develop high temperatures until the seventh day. In the control groups one animal developed fever on the fourth day, one on the sixth and two on the seventh.

Immunized animals that became infected following challenge of immunity tended to develop titres much higher than those of non-immunized control guinea pigs. Control animals developed serological titres of 1:1000 as a result of infection while titres of 1:10,000

TABLE XIV

TEMPERATURE RESPONSE AND AGGLUTINATION-LYSIS ANTIBODY TITRES OBTAINED THREE WEEKS AFTER CHALLENGE IN GUINEA PIGS IMMUNIZED WITH SEVERAL KILLED SUSPENSIONS OF L. POMONA

Group number	Antigen	Animal number	Temperature response	AL titre[a]	Group number	Antigen	Animal number	Temperature response	AL titre[a]
1B	1	961	+	1,000	5B	5	981	+	1,000
		962	+	100			982	+	100,000
		963	-	-			983	+	100,000
		964	+	100			984	+	10,000
		965	-	100			985	-	1,000
2B	2	966	-	100	6B	6	986	-	10
		967	+	10,000			987	+	10,000
		968	-	100			988	-	10
		969	-	10			939	+	100,000
		970	-	-			990	-	1,000
3B	3	971	-	1,000	7B	7	991	+	100,000
		972	-	1,000			992	-	1,000
		973	-	100,000			993	-	100
		974	+	10,000			994	-	10,000
		975	-	1,000			995	+	100,000
4B	4	976	-	10,000	8B	Control	996	+	1,000
		977	+	100,000			997	+	1,000
		978	-	10			998	+	1,000
		979	+	10,000			999	-	1,000
		980	+	10,000			1000	+	1,000

and 1:100,000 occurred in previously immunized animals infected through challenge. The exception again was group 1B in which titres of infected animals approximated those of the control group. It was concluded that antigen 2 stimulated most protection while antigens 1, 4 and 5 stimulated little or none. Indeed, the early temperature response among group 1 guinea pigs suggested increased susceptibility.

An appraisal of results of serological tests as listed in Table XIII and of effective immunity as shown in Table XIV suggests a correlation between the two. It would appear that a leptospiral antigen such as antigen 1 which stimulated little or no serological response demonstrable in the agglutination-lysis test, also showed the least in vivo protective ability against infection. Conversely a good antigen such as antigen 2 as evidenced by the serological tests also stimulated best protection.

Calves.- Two of the antigens which had been used in the guinea-pig experiment were selected for experimental use in calves. These were antigen 1 which appeared to stimulate the least protective antibody in guinea pigs and antigen 2 which gave the best results.

Ten calves approximately four months old were purchased from local farmers. These were divided at random into 3 groups. Groups 1 and 2 contained four animals each and group 3 contained two. Each of the four calves in group 1 received 5 ml. of antigen one injected subcutaneously in the neck. Group 3 animals were retained as controls. Three weeks after immunization blood was collected from all animals for serological testing. All calves were then exposed to leptospirosis

by the subcutaneous injection of 2 ml. of defibrinated calf blood drawn at height of temperature from a calf infected with L. pomona. Leptospirae were demonstrated in the challenge material by dark-field microscopy. An attempt was made to titrate the infectivity of this material but was only partially successful. Calves available for this purpose were under one month old. The subcutaneous inoculation of 2 ml. of defibrinated infective calf blood, used undiluted and in the 1:10 and 1:100 dilutions, resulted in death of the animals between the 9th and 11th post-inoculation days. The calves developed high temperatures, anaemia, icterus and haemoglobinuria before death. Dark-field examination of blood revealed the presence of leptospirae. While the degree of infectivity had not been established the material was shown to be highly virulent.

Following challenge of the immunity temperatures were recorded twice daily for ten days and once daily for the succeeding eight days. The temperature record is presented in Table XV. Both control calves revealed increased temperature up to 107.8°F. between the third and seventh post-challenge days. Brief temperature increases occurred in animals of groups 1 and 2 on the fifth post-challenge days.

Blood was collected for the agglutination-lysis test after approximately 3, 4, 8, 17, 19 and 46 weeks. Results of these tests are presented in Table XVI. None of the animals developed detectable agglutination-lysis titres after immunization. Following challenge the titres of the early bleedings of animals in group 1 ranged from 1:100 to 1:1000 and later settled at 1:100. Serum titres of animals in

TABLE XV

POST-CHALLENGE TEMPERATURE RESPONSE IN IMMUNIZED CALVES

Group no.	Calf no.	Temperature recorded on days 3 to 7 inclusive[a]									
		3		4		5		6		7	
		AM	PM	AM	PM	AM	PM	AM	PM	AM	PM
1	71	102.5	102.6	101.8	102.8	102.6	103.8	102	103.1	101.3	103.2
	56	103.2	102.6	102.9	102.9	102.9	104	103.1	103	102.1	102.8
	58	103	103.2	102.8	103.8	102.5	104.4	102.5	103.5	102.1	103.3
	53	103.4	102.4	102.8	103.4	103.5	104.3	103.4	103.7	102.6	102.5
2	52	101.9	103.3	102.7	103.3	102.4	104.1	103.2	103.3	102.2	102.8
	57	103.2	103	104.2	103.8	103	105	104.6	104.4	102.5	102.9
	55	102.7	102.9	102.5	103.5	102.3	104.8	103.2	103.4	102.2	103.4
	54	102.9	102.9	103.3	103.2	102.4	103.7	103.2	103.7	102	102.4
3	51	101.8	103	102.4	103.9	104.8	105.2	103.2	104	103.9	104.6
	72	104.2	103.8	106.8	107.8	107.4	106.8	104.4	105	104.1	105.1

[a]Temperatures for days 1-2 and 8-18 were within the normal range.

TABLE XVI

RESULTS OF AGGLUTINATION-LYSIS TESTS OF CALVES' SERA COLLECTED AFTER IMMUNIZATION AND AT INTERVALS AFTER CHALLENGE

Group number	Calf number	Agglutination-lysis titres[a]						
		21 days post-immunization	Days post challenge					
			19	28	54	117	130	325
1	71	-	100	100	10	100	100	10
	56	-	1000	100	100	100	100	10
	58	-	100	100	1000	100	100	10
	53	-	100	100	1000	100	100	10
2	52	-	100	100	100	10	10	-
	57	-	10	10	100	10	10	-
	55	-	10	100	10	10	10	-
	54	-	10	100	100	10	10	-
3	51	-	1000	1000	1,000,000	10,000	10,000	1000
	72	-	1000	100	1000	1000	1000	100

[a]Titres are expressed as the reciprocal of the highest serum dilution giving a positive reaction.

group 2 were generally a ten-fold dilution lower, being 1:10 to 1:100 in early bleedings and falling to 1:10 in later bleedings. Tests performed 11 months after challenge revealed group 1 animals to have agglutination-lysis titres of 1:10 while no antibody was detected in animals of group 2. Among the control animals one quickly developed a titre of 1:1,000,000 and was 1:10,000 after five months. This had dropped to 1:1000 after 11 months. The other control calf maintained a steady titre of 1:1000 for more than five months but this had decreased to 1:100 11 months after challenge of immunity.

The development of the low agglutination-lysis titres in the immunized animals following challenge raised the question as to whether or not these titres were simply a secondary response to reintroduction of an antigen. In order to test this possibility four calves about three months old were immunized using antigens 1 and 2, two animals for each antigen. Unfortunately one animal of the second group died of Claustridium chauvaei infection about three weeks later. The three surviving calves were bled for an agglutination-lysis test three weeks after immunization. These tests gave negative results and the calves were re-inoculated with a second dose of the suspension used previously. After a further three weeks the agglutination-lysis test was again performed on their sera. Results of this experiment are tabulated in Table XVII and show a slight antibody response in the animal given two inoculations of antigen 2 but none in either of the calves which received antigen 1. These results may be compared with those obtained earlier in calves inoculated with antigens 1 and 2 followed three weeks later by challenge

TABLE XVII

AGGLUTINATION-LYSIS TITRES THREE WEEKS AFTER IMMUNIZATION OF CALVES USING TWO INOCULATIONS WITH A THREE WEEK INTERVAL

Calf number	Antigen	AL Titre after 1st dose	AL Titre after 2nd dose
506042	1	-	-
506044	1	-	-
506043	2	Died	
506045	2	-	1:10

of infective material as shown in Table XVI. Antigen 1 injected twice at a three-week interval did not stimulate a demonstrable serological response but a single inoculation followed by infective material resulted in serum antibody titres of 1:100 to 1:1000. The serum of the calf that was inoculated twice with antigen 2 exhibited a low agglutination-lysis titre of 1:10. The calves that had received infective material developed similar low titresof 1:10 to 1:100. Since there was considerably more antigenic material in the second 5 ml. inoculation of antigen than in the 2 ml. of infective blood used for challenge, it was concluded that replication of leptospirae must have occurred in the animals of group 1 to account for their serological titres.

Reactivity to Fractions of Leptospira Pomona

Fractions of L. pomona, suspensions of the organism and by-products of the culture as well as culture medium were compared for immunological reactivity. The material investigated can be divided into 7 groups as follows:

1. Water insoluble fraction of an alcohol extract of L. pomona organisms
2. Water soluble fraction of an alcohol extract of L. pomona organisms
3. Culture supernatant liquor
4. Sonic vibrated leptospiral suspension in saline
5. Sonic vibrated whole culture
6. Antigen 2
7. Korthof's medium

Alcohol Extracts.- The water insoluble fraction and water soluble fractions of alcohol extracts of L. pomona were prepared according to the method of Rothstein and Hiatt (94) with slight modification. One litre of Korthof's medium was dispensed in bottles in 100 ml. amounts and inoculated with L. pomona. After nine days incubation at 30°C when growth was considered satisfactory, the cultures were killed by the addition of formalin to 0.5 per cent and were allowed to stand overnight in the refrigerator. The following day the organisms were precipitated by centrifugation in a Servall centrifuge at approximately 15,000 r.p.m. for 20 minutes at 9°C. The supernatant liquor was saved and the precipitate was washed three times by resuspending in distilled water with a needle and syringe followed by centrifugation. After the final washing the sediment was resuspended in 10 ml. distilled water and sonic vibrated in a Raytheon 10 KC magnetostrictive oscillator. Sonic vibration was repeated a further ten minutes after addition of 20 ml. absolute ethanol. The material was then placed in the shaker overnight at 9°C. The following day it was centrifuged in the cold for 20 minutes at 15,000 r.p.m. and the supernatant fluid was saved. The precipitate was resuspended in 12 ml. of distilled water and 36 ml. absolute ethanol was added to make a 70 per cent alcohol concentration. The material was again shaken overnight in the cold and then centrifuged to obtain the supernatant extract. The extraction was repeated a third time following which the three supernatant extracts were placed in a cellophane dialysis sac and dialysed against running tap water for 48 hours. The extract became very milky in appearance during this time. Pervaporation was carried out for 18 hours

to reduce the volume followed by dialysis against six changes of distilled water for two days. Centrifugation at 15,000 r.p.m. for one hour yielded a clear water-soluble supernatant referred to as "precipitinogen" (94) and a water insoluble sediment referred to as "complement-fixing antigen" (94). This sediment was resuspended in normal saline to a volume of 25 ml. Merthiolate to 1:10,000 final concentration was added to both fractions as a preservative.

Culture liquor.- This was obtained by growing a culture of L. pomona in Korthof's medium for three weeks. The leptospirae were removed by centrifugation at 15,000 r.p.m. for one hour in the cold followed by filtration through a Seitz D8 sterilflo pad. Sterility was checked by dark-field microscopy and by cultural techniques and the liquor was found to be sterile.

Sonic Vibrated Saline Suspension of Leptospirae.- Thegrowth which was harvested in the preparation of the culture liquor was employed. The sediment was washed once with normal saline and resuspended in 5 ml. normal saline to make a slightly cloudy suspension. This material was sonic vibrated for ten minutes in a Raytheon 10KC magnetostrictive oscillator. Dark-field examination and cultural methods revealed it to be sterile.

Sonic Vibrated Whole Culture.- This was prepared from a 12-day, well grown culture which was sonic vibrated for ten minutes in a Raytheon 10KC magnetostrictive oscillator. Leptospirae could not be demonstrated by dark-field examination or cultural methods.

Korthof's Medium.- This was Korthof's base medium containing normal rabbit serum as previously described which served as a control material.

Animals.- Fourteen guinea pigs averaging 521 gm. were divided into seven groups. Antigens were administered in 0.5 ml. amounts intraperitoneally. Blood was collected 14 days post-inoculation for serological testing. The results obtained are summarized in Table XVIII. It will be noted that neither the water soluble nor the water insoluble fractions of the alcohol extract of L. pomona stimulated detectable antibodies in the agglutination-lysis test. Low antibody titres of 1:10 to 1:100 were stimulated by supernatant culture liquor alone. The three suspensions of leptospirae were equally effective in stimulating titres of 1:1000.

TABLE XVIII

SEROLOGICAL RESPONSE OF GUINEA PIGS TO INOCULATION WITH L. POMONA AND FRACTIONS THEREOF, AS INDICATED BY THE RESULTS OF THE AGGLUTINATION-LYSIS TEST

Animal number	Inoculum	Agglutination-lysis titre[a]
770	Water insoluble fraction	-
797	" " "	-
799	Water soluble fraction	-
789	" " "	-
800	Culture liquor	100
787	" "	10
798	Sonic vibrated leptospirae in saline	1000
792	" " " "	1000
774	Sonic vibrated whole culture	1000
773	" " " "	1000
779	Antigen 2	1000
794	" "	1000
782	Karthof's medium	-
784	" "	-

[a]Titres are expressed as the reciprocal of the highest serum dilution giving a positive reaction

PART III

Tests of Infection or Immunity

In the literature review it has been pointed out that leptospirosis due to L. pomona in large animals is often manifested by certain clinical signs. However, this is not constant and the disease may be asymptomatic as it usually is in laboratory animals. Other criteria of infection or immunity must be employed. These are temperature response, demonstration of the organism and antibody formation.

Temperature Response

Guinea Pigs.- The temperature curve of guinea pigs infected with leptospirosis has a definite pattern. Tables I, II and III show that inoculated animals maintained normal temperatures for five to nine days. The duration of the incubation period depended somewhat upon the size of the inoculum, those animals which received the larger doses having shorter incubation times. Onset of temperature was abrupt. From normal temperatures in the 103°F range on the final day of incubation there was usually a sharp rise to approximately 105°F on the first day of temperature. Peak temperatures were usually reached on the second day of fever and reached 105.5 to 106°F. Fever was usually of three days' duration. There was then a sudden drop to normal or near normal for one day after which many animals developed a second brief temperature rise of one day duration to approximately 104°F before temperatures returned to normal permanently. All guinea pigs which became infected revealed a temperature response.

Calves.- Table XIX presents the temperature charts of calves which had been infected with L. pomona for various purposes during the experimental work. In these animals response was usually definite with a sharp rise of 2° to 4°F on the first day of temperature. The length of the incubation period varied from as little as two days up to nine days and showed some relationship to the number of leptospirae in the inoculum and to the age of the animal. Calf 42305, which was only three weeks old, developed fever on the third day following subcutaneous injection of 8 ml. of whole culture of L. pomona grown in Korthof's medium. Calf 506033, which was a week older, revealed fever on the fourth day following its inoculation with 10 ml. of whole culture. Calves 506107 and 506108 which were two months old received diluted culture containing only 12,000 organisms and temperature rise occurred on the seventh post-inoculation day. When virulent calf blood was used as inoculum fewer organisms were introduced than when culture was used but the virulence of the organisms appeared to be greater in this state than in the artificial medium. Calves 506035, 24934 and 24935 which were approximately three weeks old received various dilutions of infective blood and died of acute leptospirosis. Calf 506035 had received 2 ml. of undiluted blood and died on the ninth post-inoculation day before showing fever. The other two animals received 1:10 and 1:100 dilutions of infective blood and temperature response occurred on the sixth and eighth post-inoculation days respectively. These animals died on the twelfth day following exposure.

Only two calves of 15 which developed infection failed to show pyrexia. These were calf 506035 that died suddenly of leptospirosis and

TABLE XIX

TEMPERATURE RECORDS OF CALVES EXPERIMENTALLY INFECTED WITH L. POMONA

Calf	Age	Inoculum	Post-inoculation days 1	2	3	4	5	6
24935	17 days	2 ml.1/100 Dil. Calf Blood	101.5	101.4	101.4	103.8	101.2	101.6
24934	25 days	2 ml. 1/10 Dil. Calf Blood	102.6	102.2	102.2	101.8	101.8	105.6
506035	3 wk.	2 ml. Calf Blood	102	102.5	102.2	100	102.7	102.4
506034	1 mo.	2 ml. Calf Blood	101.6	101.8	102.6	104.4	107	105.9
94173	4 mo.	2 ml. Calf Blood	102.2	101.6	103.6	104.6	104.8	104.8
94170	4 mo.	2 ml. Calf Blood	101.2	101.5	101.8	101.3	102.6	104.4
94172	4 mo.	2 ml. Calf Blood	102.6	100.4	103.8	107.8	106.8	105
94151	4 mo.	2 ml. Calf Blood	102.4	102.8	103	103.9	105.2	104
24926	2 mo.	1 ml. Guinea Pig Blood 1.P	102.6	102.4	102.2	102	102.6	102.8
24929	2 mo.	1 ml. Guinea Pig Blood 1.P	103.3	103.6	103.3	102	103	102.8
506033	1 mo.	10 ml. Culture	102.8	102	103.2	104.8	106.2	104
42305	3 wk.	8 ml. Culture	102.9	102.3	104.5	105.6	104.9	102.6
506104	2 mo.	12,000 leptospirae(culture)	101.8	101	101.6	102.2	101.1	102
506107	2 mo.	12,000 leptospirae(culture)	101.6	102.2	102.2	102.4	101.6	102.4
506108	2 mo.	12,000 leptospirae(culture)	102	101.6	102.6	102.3	101.8	102.6

(continued)

TABLE XIX.- TEMPERATURE RECORDS OF CALVES EXPERIMENTALLY INFECTED WITH L. POMONA (continued)

Calf	Age	Inoculum	Post-inoculation days 7	8	9	10	11	12
24935	17 days	2 ml. 1/100 Dil. Calf Blood	101	105	104.8	105	105.8	Moribund
24934	25 days	2 ml. 1/10 Dil. Calf Blood	104.7	105	102.8	102.5	101.5	Dead
506035	3 wk.	2 ml. Calf Blood	101.4	102.7	Died			
506034	1 mo.	2 ml. Calf Blood	104.6	105	103.8	104.4	103.7	103.5[a]
94173	4 mo.	2 ml. Calf Blood	102.8	101.8	101.2	101.2	103	101.5
94170	4 mo.	2 ml. Calf Blood	104.1	103.2	101.8	102.7	103.5	102.2
94172	4 mo.	2 ml. Calf Blood	105.1	103	103.4	103.3	102.5	100.5
94151	4 mo.	2 ml. Calf Blood	104.6	102	103.2	102.6	102.6	102
24926	2 mo.	1 ml. Guinea Pig Blood 1.P	103.5	104.4	105	103	103.6	105.0[b]
24929	2 mo.	1 ml. Guinea Pig Blood 1.P	102.4	103.8	104	104.3	101.8	103.7
506033	1 mo.	10 ml. Culture	103.4	103.6	101.4	101.6	102.5	102
42305	3 wk.	8 ml. Culture	101.8	103.2	103.5	103.5	103.4	103.6
506104	2 mo.	12,000 leptospirae (culture)	101.8	102.2	103.8	103.6	102.3	102.2
506107	2 mo.	12,000 leptospirae (culture)	104	103.8	103	102.6	102.2	102
506108	2 mo.	12,000 leptospirae (culture)	104.6	104.2	105.6	102.2	102.2	102

[a]Temperature was 104°F on days 13 and 14.
[b]Temperature was 104.6°F on day 13.

calf 506104 which became a urinary shedder. Duration of fever in the remaining 13 animals was usually one to three days but two animals, numbers 506034 and 24926, showed long irregular temperature responses of up to 11 days' duration.

Swine.- Temperature records of four experimentally infected swine are given in Table XX. One animal failed to develop pyrexia. The other three revealed fever on the sixth to eighth post-inoculation days. Peak temperatures of 105 to 105.6°F were reached. Duration of temperature varied from three to nine days.

Temperature response would appear to be a constant finding in infected guinea pigs. Similar response was the rule in calves and young pigs but occasional exceptions occurred.

Demonstration of Leptospirae

Three techniques were used for this purpose, i.e. the direct examination of tissues and fluids by dark-field microscopy, the cultivation of the organisms and their demonstration by means of fluorescent microscopy.

Dark-field Examination of Blood.- Table XXI gives the results of dark-field examination of ten blood samples collected from calves at the height of temperature. Living leptospirae were demonstrated in seven of them. Similar examinations conducted in guinea pigs revealed the organisms in the blood of only one animal of six examined. These findings suggest that leptospirae are more numerous in the blood of infected calves than in that of guinea pigs. There is no doubt that leptospirae are always

TABLE XX

TEMPERATURE RECORDS OF SWINE EXPERIMENTALLY INFECTED WITH L.POMONA

Pig	Age	Inoculum	Post - inoculation days[a] 6	7	8	9	10	11	12	13	14	15
24789	2 mo	2 ml. culture in Korthof's medium	104.4	105	104.8	105	104.4	104	103.6	103.2	103.6	103
24791	"		103	103	103	103.2	103.4	103.1	103.4	103	103	103.2
24792	"		103.8	105	104.4	104.8	105.2	104.5	105.2	104.8	104.8	104.4
24794	"		103.6	103.6	104.4	104.8	105.6	103.8	103.1	102.4	102.8	103

[a]Temperatures during the first five days were within the normal range.

TABLE XXI

COMPARISON OF TECHNIQUES USED TO DEMONSTRATE INFECTION WITH L.POMONA IN CALVES

Calf number	Temp. response	Blood culture		Darkfield urine	Darkfield blood	Animal inoculation	Serum AL titre
		Korthof	Fletcher				
24926	+	-	-	-	N.D.	+	1,000
24929	+	+	-	-	N.D.	+	1,000
506033	+	+	+	N.D.	+	+	1,000
506034	+	+	+	-	-	+	1,000
506035	-	+	+	-	+	+	N.D.
24934	+	+	+	-	+	+	N.D.
24935	+	+	+	-	+	+	N.D.
94173	+	+	-	-	-	+	1,000
94170	+	-	-	-	-	-	1,000
94151	+	+	-	+	+	N.D.	10,000
94172	+	+	-	+	+	N.D.	1,000
42305	+	+	+	-	+	N.D.	1,000
506104	+	+	N.D.	+	N.D.	N.D.	10,000
506107	+	+	N.D.	+	N.D.	N.D.	100
506108	+	+	N.D.	-	N.D.	N.D.	1,000

present in the blood of guinea pigs at the height of temperature. The data to be presented later in a discussion of the results of cultural work support this hypothesis.

Dark-field Examination Urine.- Table XXI shows the results of dark-field examination of calves urine. Of the 14 samples examined, leptospirae were found in only four. The positive samples had been collected 30 to 34 days post-infection. Seven of the negative samples were examined three to 12 days post-infection, one was examined 30 days post-infection and two were examined 46 and 51 days after infection.

Urine samples from four swine experimentally infected with L. pomona were collected at intervals and results of dark-field examinations are shown in Table XXII. Leptospirae could not be demonstrated in the urine 16 days after inoculation of the animals. Two pigs were shedding leptospirae in their urine after 23 days and all four were doing so after 42 days.

These findings suggest that urine examinations should be commenced about two weeks after infection and should be repeated at intervals for at least two months.

Culture.- As mentioned in Part I, positive blood cultures in Korthof's medium were made from all 12 guinea pigs which were bled at the height of temperature. Cultures were also made from the blood of 12 calves at the height of fever due to leptospirosis. Both Korthof's fluid medium and Fletcher's semi-solid medium were employed. Table XXI shows that isolations were made from the blood of ten calves using Korthof's medium and from six using Fletcher's medium. These

TABLE XXII

RESULTS OF DARK-FIELD EXAMINATION OF URINE SAMPLES FROM SWINE EXPERIMENTALLY INFECTED WITH L. POMONA

Pig number	Post-inoculation day of temp.	Post inoculatikn day of D.F. examination		
		16	23	42
24789	6	-	+	+
24791	-	-	+	+
24792	7	-	-	+
24794	8	-	-	+

findings indicate that culture of blood during the febrile stage is a reliable method for demonstrating L. pomona. In this instance the use of Korthof's medium appeared to give superior results.

An extension of this work to the study of cultural techniques for the isolation of leptospirae from swine kidneys was made. Pig kidneys were obtained from an abattoir and cultures were made within two hours of collection. Of the 36 kidneys examined, 19 contained lesions resembling those seen in cases of leptospirosis. Table XXIII reveals that 14 isolations were made from the 19 kidneys which exhibited gross lesions of leptospirosis and from four of the 17 kidneys which appeared to be normal. The table indicates the effect of size of the inoculum used in seeding the media upon the success of isolations. When 1 ml. of a 1:10 ground tissue suspension was used as inoculum in Korthof's medium only one successful isolation was made. When the inoculum was diluted 1:100 and 1:1000 there were 12 and 13 isolations respectively. The semi-solid medium was inoculated with three drops of the 1:10 material and 18 isolations were made. An inhibitory effect of the kidney tissue in the inoculum appeared to be reduced by dilution and when this was done semi-solid medium gave superior results.

Fluorescent Antibody Technique.- Table XXIV presents a comparison of the efficacy dark-field and fluorescent microscopy in the detection of leptospirae in urine and kidney tissue of experimentally infected animals. Of the nine animals which were shown to be infected by cultural and serological techniques leptospirae were found in the kidneys of six by dark-field microscopy and in five by the fluorescent antibody technique.

TABLE XXIII

THE EFFECT OF SIZE OF INOCULUM AND OF MEDIA USED UPON THE ISOLATION OF *L. POMONA* FROM FIELD SPECIMENS OF SWINE KIDNEYS

Swine kidney number	Dilution of inoculum in Korthof's medium			Fletcher's medium
	1:10	1:100	1:1000	
1	-	+	-	+
2	-	+	-	+
3	-	+	+	+
5	-	+	+	+
6	-	-	-	-
9	-	-	+	+
10	-	-	-	-
13	-	-	-	-
14	-	-	-	-
17	-	+	+	+
18	+	+	+	+
21	-	-	+	+
22	-	-	+	+
25	-	-	-	-
26	-	-	-	-
29	-	-	+	+
30	-	-	+	+
33	-	+	+	+
34	-	+	+	+
23[a]	-	+	-	+
24[a]	-	+	+	+
31[a]	-	+	-	+
32[a]	-	+	+	+

[a]Four of 17 swine kidneys examined which appeared normal grossly but from which leptospirae were isolated.

TABLE XXIV

A COMPARISON OF DARK-FIELD AND FLUORESCENT MICROSCOPY IN THE DETECTION OF LEPTOSPIRAE IN KIDNEY TISSUE AND URINE

Animal identification	Serological status	Kidney			Urine	
		Culture	DF	FL	DF	FL
Calf 506103	-	-	-	-	-	±
Calf 506104	+	+	++	++	++	++
Calf 506107	+	+	++	+	-	±
Calf 506108	+	-	-	-	-	±
Hamster 6	+	+	+	++	N.D.	N.D.
Hamster 7	+	+	++	++	N.D.	N.D.
Hamster 8	+	+	++	++	N.D.	N.D.
Hamster 12	+	+	-	-	N.D.	N.D.
Rabbit B	+	-	+	-	N.D.	N.D.
G. Pig 808	+	+	-	-	N.D.	N.D.

± a few indefinite structures.
N.D. not done.

Successful cultivation of the organism was possible with seven of these kidneys. Plates 1 and 2 show L. pomona in culture medium as demonstrated by fluorescent microscopy. Plates 3 to 6 picture the organisms by this technique in urine and kidney specimens from one of the experimentally infected calves.

Reference has already been made to culture studies of swine kidneys obtained from an abattoir. Dark-field and fluorescent microscopic examinations were made of these tissues. Of the 18 kidneys proven infected by culture methods, leptospirae could be demonstrated in three by dark-field examination and in two of these by the fluorescent antibody technique. A problem encountered using fluorescent stain was the presence of confusing structures which could not be definitely identified. These were found in seven of the infected kidneys and in two normal kidneys.

Demonstration of Antibodies

The three serological tests employed during the course of the experiments were compared in guinea pigs and calves.

Guinea Pigs.- Table V presents the results of agglutination-lysis, complement-fixation and plate-agglutination tests of the sera of 20 guinea pigs infected with L. pomona organisms.

Plate-agglutination Test.- Antibody titres as revealed by the plate-agglutination test performed on the 15th post-inoculation day varied widely. Some sera reacted in the undiluted state whereas others gave reactions in the 1:80 dilution. In all but three animals the serum titres had reached a peak on the 15th day. In the three exceptions, numbers 761,

768 and 753, temperature response to infection had been delayed two to four days with a concomitant delay in the appearance of antibodies. After infection subsided the plate-agglutination titres fell rapidly and 60 days later the sera of all but two of the guinea pigs reacted only in the undiluted state. The two exceptions gave reactions in the 1:10 serum dilution.

Agglutination-lysis Test: Agglutination-lysis titres rose soon after inoculation of the animals and in the 15 day post-inoculation samples 12 of the 20 guinea pig sera had reached maximum titres. Of these 12, two reacted in the 1:10,000 and ten in the 1:1000 dilution. Seven of the remaining eight injected animals had reached maximum and comparable titres by the 30th post-inoculation day. Sixty days after inoculation the serum antibody titres of all animals had levelled off at about a 1:1000 dilution.

Complement-fixation Test: Antibodies were not demonstrated by the complement-fixation test as soon after inoculation as by the other two methods. Sera from only five animals showed low titres up to the 1:20 dilution when collected 15 days post-inoculation. In the 30-day test the sera of all infected animals showed titres which varied from 1:10 to 1:320. In most instances serum titres were higher in the 60-day tests than in those made after 15 and 30 days.

Calves.- Blood was collected at intervals from the ten calves used in the experiment undertaken to determine the immunological reactivity of calves to killed suspensions of L. pomona (Part II). Two groups of four animals each had been immunized with the suspensions shown to be

the most and the least effective respectively in protecting guinea pigs against experimental infection. These eight immunized calves and two normal control animals were subsequently injected with infective material. Agglutination-lysis, complement-fixation and plate-agglutination tests were performed on their sera at intervals for 325 days following challenge of immunity. Table XXV summarizes the results obtained. Three of the four calves which had received the poorer antigen showed low but significant antibody titres in all three types of test. The agglutination-lysis titres were approximately 1:100. The complement-fixation titres of three of the calves ranged from 1:10 to 1:160 and in the plate-agglutination test from the undilute to 1:10 dilutions. These titres were maintained throughout the period of observation except for the 325th day test when activity in the complement-fixation and plate-agglutination tests had disappeared. Agglutination-lysis titres of 1:10 persisted in this test.

The results obtained with sera of animals in group 2 which had received the better antigen contrasted sharply with those of group 1. Although the agglutination-lysis antibody titres of both groups were of about the same order, 1:10 to 1:1000 in the 28 and 54 day tests, in group two they had decreased to 1:10 as compared with 1:100 by 117 and 130 days and had disappeared completely by the 325th day. No reactions were demonstrated by the complement-fixation test in any of the bleedings from group 2 calves and only two low grade reactions were obtained in the plate-agglutination tests performed on the 28th day post-challenge.

In the two control calves the serum antibody titres in all three tests tended to be much higher and to persist longer than in the immunized

TABLE XXV

COMPARISON OF AGGLUTINATION-LYSIS (A.L.), COMPLEMENT-FIXATION (C.F.) AND PLATE-AGGLUTINATION (P.A.) TITRES OF SERA COLLECTED AT INTERVALS FROM CALVES EXPOSED TO LEPTOSPIRAE FOLLOWING IMMUNIZATION

Group	Calf number	Days after inoculation: 28 A.L.	28 C.F.	28 P.A.	54 A.L.	54 C.F.	54 P.A.	117 A.L.	117 C.F.	117 P.A.	130 A.L.	130 C.F.	130 P.A.	325 A.L.	325 C.F.	325 P.A.
1	75	100	-	10	10	-	0	100	-	-	100	-	-	10	-	-
	56[a]	100	40	10	100	80	10	100	80	0	100	80	0	10	-	-
	58	100	10	0	1000	40	0	100	10	0	100	20	0	10	-	-
	53	100	40	10	1000	160	10	100	40	0	100	40	0	10	-	-
2	52	100	-	0	100	-	-	10	-	-	10	-	-	-	-	-
	57	10	-	-	100	-	-	10	-	-	10	-	-	-	-	-
	55	100	-	0	10	-	-	10	-	-	10	-	-	-	-	-
	54	100	-	-	100	-	-	10	-	-	10	-	-	-	-	-
3	51[a]	1000	160[b]	40	1,000,000	160[b]	160	10,000	160[b]	160	10,000	160[b]	40	1,000	160[b]	10
	72[a]	100	160[b]		1,000	160[b]	0	1,000	160	0	1,000	160	0	100	-	-

[a] Leptospirae demonstrated in the urine.
[b] End point not reached.

animals. In the agglutination-lysis test, titres reached the 1:1,000 and 1:1,000,000 levels respectively. The complement-fixation titres were in excess of 1:160 which was the highest dilution employed in the tests. The plate-agglutination titres were 1:40 and 1:160 respectively.

Of the three serological tests employed the agglutination-lysis test is the one most generally used. It has the advantage of being highly sensitive,and of detecting reactivity soon after infection and for months or even years after recovery of the animal. However, it is serotype-specific, reacting only when the antigen and serum antibody in the test are homologous. This can be a disadvantage when the object is to establish whether or not an animal has had previous experience with various leptospiral serotypes. For this purpose the complement-fixation test with an alcohol extract antigen which is group reactive offers an advantage. Both the agglutination-lysis test which requires the use of live culture and the complement-fixation test are laborious techniques and for this reason the plate-agglutination test is usually favored by laboratories with limited facilities.

The Demonstration of Antibodies in Kidney Tissue Suspensions

The histopathological changes induced in swine kidneys by infection with L. pomona have been described by Burnstein and Baker (28), Langham et al (123) and Boulanger et al (49). Focal infiltrations of lymphocytes, plasma cells and macrophages are characteristic and account for the gross appearance of pale foci scattered throughout the cortex. Kidneys showing the typical spotted appearance are pictured in plates

7 and 8. Since these cells of the reticulo-endothelial system are recognized as of primary importance in the production of antibody it would be anticipated that kidneys having well developed lesions of leptospirosis might contain high levels of antibody for leptospirae.

In a preliminary experiment two swine kidneys were obtained which contained numberous typical gross lesions of leptospirosis and from which leptospirae were demonstrated by dark-field examination. Two normal kidneys were used for control purposes. Tissue suspensions were prepared from each kidney by grinding 1 gram of tissue in physiological saline in a Tenbroeck grinder. The volume was made up to 10 ml. with physiological saline. The suspensions were centrifiged in the cold in a Servall centrifuge at approximately 15,000 r.p.m. for one hour. The supernatant fluid was removed from below the surface fatty layer by means of a Pasteur pipette. An agglutination-lysis test was performed using the material undiluted and in five-fold dilutions from 1:2 to 1:1250. Table XXVI shows that titres of 1:50 were obtained using the suspensions of infected kidneys. Positive reactions were typical of those seen when serum is tested. Suspensions of normal kidney produced some diminution in numbers of leptospirae when undiluted suspension was used in the test but agglutination was not observed.

A second experiment was conducted utilizing kidneys from animals used in Part I studies of the relative susceptibility of various animal species to leptospirosis. The kidney tissue from these animals had been stored at -20°C. Suspensions were made of kidneys from guinea pigs, rabbits, swine and calves. These were prepared as previously described with the exception that centrifugation was done in an International centrifuge

TABLE XXVI

RESULTS OF AGGLUTINATION-LYSIS TESTS USING SUSPENSION OF SWINE KIDNEY IN PLACE OF SERA

Kidney	Number	Dilution of suspension					
		0	1:2	1:10	1:50	1:250	1:1250
Infected	1	4	4	3	2	-	-
Infected	2	4	4	3	2	1	-
Normal	3	3[a]	-	-	-	-	-
Normal	4	1[a]	-	-	-	-	-

[a]Apparent loss of density of the antigen but no agglutination noted.

model S.B.V. at 2,500 r.p.m. for one hour. Titres obtained using the kidney suspensions were compared with those of sera collected at the time of euthanasia. Table XXVII shows that kidney tissue from serologically negative guinea pigs was inhibitary in the 1:2 dilution while that from the two infected ones showed agglutination-lysis reactions in the 1:10 dilution. Serum titres of the infected guinea pigs was 1:1000. The kidney suspensions of the normal rabbits produced results similar to those of the guinea pigs but the titre of the infected rabbit kidney was 1:50 as compared with the serum titre of 1:10,000. None of the swine were infected and kidney suspensions from them compared with those of the normal guinea pigs and rabbits. Normal calf kidney did not appear to be inhibitary to leptospirae. Suspension material from one of the infected calf kidneys showed an antibody titre of 1:10. This animal had a serum titre of 1:10,000. Two other calves with serum antibody titres of 1:100 and 1:1000 had kidney suspension titres of 1:2.

A third experiment was performed to compare the serum agglutination-lysis antibody titres with those of kidney suspensions of normal and infected swine. Seven pigs approximately ten weeks old were divided at random into two groups, one of four and the other of three animals. Each of the four pigs in group 1 was injected with 2 ml. of a well-grown active culture of L. pomona. Half of the inoculum was given subcutaneously in the pectoral region and the remainder was injected intraperitoneally. Group 2 was retained as a control. Temperatures were recorded daily for 17 days. Urine samples were collected periodically for dark-field examination. The animals were kept for 52 days to

provide an opportunity for the development of renal foci of infection. Blood was collected for the agglutination-lysis test and the animals were stunned and exsanguinated. Post-mortem examinations were conducted and kidneys were saved from all animals. Kidney suspensions were prepared as previously described.

TABLE XXVII

RESULTS OF AGGLUTINATION-LYSIS TESTS PERFORMED ON SERA AND SUSPENSIONS OF KIDNEY TISSUE OF ANIMALS EXPOSED TO LEPTOSPIRAL INFECTION

Species	A L titres[a] of sera	A L titres[a] of kidney suspension
Guinea pig 808	1000	10
" " 815	1000	10
" " 810	-	2
" " 821	-	2
Rabbit Brown	10,000	50
" Black	-	2
" Grey	-	2
" Spot	-	2
Calf 506104	10,000	10
" 506107	100	2
" 506108	1000	2
" 506103	-	-
Pig 24801	-	2
" 24804	-	0
" 24806	-	2
" 24813	-	10
" 24815	-	2
" 24817	-	0
" 24825	-	2
" 24829	-	2
" 24830	-	2
" 24786	-	2

[a]Titres are expressed as the reciprocal of the highest dilution in which a positive reaction was found.

Table XXVIII summarizes the results obtained in this experiment. All four animals in group 1 were shown to be infected by demonstration of leptospirae in their urine and of homologous antibodies in their sera. Necropsy revealed nothing remarkable in any of the organs with the exception of the kidneys. Kidneys of three of the four animals in group 1 showed small pale foci throughout the cortex suggestive of leptospirosis. Kidneys of the remaining animal of group 1 and of the 3 control pigs in group 2 appeared normal. Serum agglutination-lysis titres of three of the animals in group 1 were 1:1000 while the other was 1:10,000. Sera from group 2 pigs were negative. Kidney suspensions from the infected animals showed reactions in the 1:10 dilution and those of negative animals were inhibitary in the 1:2 dilution.

TABLE XXVIII

RESULTS OF AGGLUTINATION-LYSIS TESTS PERFORMED ON SERA AND KIDNEY SUSPENSION OF SWINE INFECTED WITH L. POMONA

Group number	Animal number	AL titres of sera	AL titres of suspension
1	24789	1,000	10
	24791	10,000	10
	24792	1,000	10
	24794	1,000	10
2	24790	-	2
	24793	-	2
	24795	-	2

Definite conclusions could not be reached from these studies. However, the titres obtained with infected kidney suspensions were low and generally only five fold higher than the inhibitary effect of normal kidney tissue suspension. Such a difference could easily be accounted

for by the presence of circulating antibody in residual blood and tissue fluid.

Mechanism of the Agglutination-lysis Reaction

Although the agglutination-lysis test is widely used its mechanism is not entirely understood. For example, what is the fate of the leptospirae when they are exposed to homologous serum antibodies? Are they simply agglutinated as suggested by Lawrence (109) or do they undergo agglutination followed by lysis, or is the lysis the primary action of serum antibodies? Another question is that of the role played by complement in the serological reaction. Is the so-called lytic phenomenon a direct result of the reaction of serum antibodies or it is a secondary autolytic process following a lethal antibody reaction?

The Effect of Complement in the Agglutination-lysis Test:- Lysis in the test does not appear to be related to autolysis nor does it resemble bacteriolysis as described with other organisms such as Vibrio comma. The classical bacteriolysis requires the presence of complement but experiments (109) have indicated that it is not required in the agglutination-lysis test. To determine whether complement is needed for lysis of the leptospira tests were set up in quadruplicate using three known positive sera, three known negative sera and one diluent antigen control. Duplicate sets of tests were performed in the usual way using normal saline as serum diluent with the exception that the duplicate racks were placed in the water bath for 30 minutes at 56^{o}C to inactivate any complement that may have been present. A second set of duplicate tests of the same sera was

set up using guinea pig complement in the diluent. The complement had been titrated by the method used for the complement fixation test and was diluted with normal saline to contain at least forty five 50 per cent hemolytic units in 0.1 ml. of diluent. The duplicate racks were placed in the water bath at 56°C for 30 minutes before addition of the antigen. Results are shown in Table XXIX. The addition of complement to the diluent did not increase the sensitivity of the test nor did the inactivation of the diluted serum decrease it. It was concluded that complement is not required in the agglutination-lysis test.

Occurrence of Lysis.- When the agglutination-lysis test is performed with a serum which contains the homologous antibodies in high titre, it is usual to find a complete reaction (4+) in each of the first three or four dilutions, i.e. up to 1:10,000. Dark-field examination of slide preparations made in the usual manner from the supernatant of the mixtures reveals only occasional motile leptospirae and little debris. As serum dilutions increase more leptospirae are found and eventually a mixture is reached in which the density of organisms is about half that of the control tubes but large compact balls of leptospirae are to be seen which usually have many motile organisms radiating from their surfaces. As serum dilution further increases the large balls are replaced by occasional small loose tangles of leptospirae and the density of the suspension is not noticeably reduced as compared with the control tubes. When various sera containing homologous antibody in greater or lesser concentration are employed, the reactions described increase or decrease proportionately. Occasionally with high titre antisera slight "prozone"

TABLE XXIX

SERUM TEST RECORDS OF AN AGGLUTINATION-LYSIS TEST PERFORMED IN THE PRESENCE AND ABSENCE OF COMPLEMENT

Regular test with active serum

Serum	Serum dilution					
	1/10	1/100	1/1000	1/10,000	1/100,000	1/1,000,000
Pos. Cow	4	4	4	3	-	-
Pos. Horse	3	4	4	3	1	-
Pos. Rabbit	4	4	4	4	2	-
Neg. Pig	-	-	-	-	-	-
Neg. Cow	-	-	-	-	-	-
Neg. G. pig	-	-	-	-	-	-
Antigen control	-					

Serum dilutions inactivated

Serum	Serum dilution					
	1/10	1/100	1/1000	1/10,000	1/100,000	1/1,000,000
Pos. Cow	4	4	4	2	-	-
Pos. Horse	3	4	4	3	-	-
Pos. Rabbit	4	4	4	4	2	-
Neg. Pig	-	-	-	-	-	-
Neg. Cow	-	-	-	-	-	-
Neg. G. pig	-	-	-	-	-	-
Antigen control	-					

TABLE XXIX.- (continued)

Test with active guinea pig serum complement

Serum	Serum dilution					
	1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000
Pos. Cow	4	4	3	2	-	-
Pos. Horse	4	4	4	3	1	-
Pos. Rabbit	4	4	4	3	2	-
Neg. Pig	-	-	-	-	-	-
Neg. Cow	-	-	-	-	-	-
Neg. G. pig	-	-	-	-	-	-
Antigen control	-					

Test with guinea pig serum inactivated

Serum	Serum dilution					
	1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000
Pos. Cow	4	4	4	2	-	-
Pos. Horse	4	4	4	4	2	-
Pos. Rabbit	4	4	4	4	3	1
Neg. Pig	-	-	-	-	-	-
Neg. Cow	-	-	-	-	-	-
Neg. G. pig	-	-	-	-	-	-
Antigen control	-					

reactions are noted in which the reaction in the first tube is 2+ and 3+ but is succeeded by 4+ reactions in greater dilutions. In the order of dilutions, that is from lower to higher, lysis appears to occur in the lower dilutions followed by agglutination in the higher ones.

The disappearance of leptospirae in the positive dilutions of the test has generally been considered to be due to lysis of the organisms. Lawrence's experiments (109) in which he immobilized leptospirae in gelatin or in agar and exposed them to antibody failed to provide evidence that individual leptospirae are really lysed. He supposed that lysis could occur only after clumping, or more likely, that lysis did not occur at all and that organisms were still present in densely compacted "lysis balls".

To determine if lysis always occurred following agglutination a lysis test was set up in the usual manner using a homologous serum and employing sterile technique. Following incubation of the test one dilution close to the end titre of the serum was found to contain large lysis balls. A slide preparation of this dilution was carefully sealed on all sides using melted paraffin. The location of several lysis balls was noted. The preparation was stored in a dark, humid chamber at room temperature and was withdrawn at intervals for examination.

During the first two hours no change was noted. Numerous leptospirae protruding from the lysis balls were actively motile and free organisms appeared normal. The following day all leptospirae were non-motile but were present as before. During the succeeding week individual free leptospirae and those protruding from the clumps became

thinner and more difficult to see. The balls contracted slightly and gradually resembled large granules. These granules were still present after two months. The gradual fading of leptospirae in this preparation over a period of several days did not resemble the rapid disappearance seen in the agglutination-lysis test within four hours. In addition, the so-called "lysis balls" did not lyse. It was concluded that lysis does not necessarily follow agglutination.

It is generally known that leptospirae inactivated by heat or chemicals do not lyse. The possibility was considered that lysis depended upon the autolytic effect of cell enzymes which were destroyed by the usual methods of inactivation. Gamma irradiation of leptospirae was investigated as a possible means of inactivation without destruction of these enzymes.

One tube of actively growing culture of L. pomona in Korthof's medium and one of uninoculated medium were exposed to gamma radiation for each of six dose levels ranging from 50,000 to 1,000,000 rad. Irradiation was accomplished by means of a "gammacell 220" unit of the Atomic Energy of Canada, Limited. Dark-field examination of irradiated cultures showed that they were not killed immediately even at the high dose levels which caused the glass of the tubes to turn brown and death of the cultures could be studied. The appearance produced was that of accelerated aging. The normal rapid spinning of the organisms and progression through the medium were replaced by occasional twitching or by short sporadic attempts to spin unaccompanied by progression. Amotile leptospirae gradually predominated in the culture and beaded forms became numerous. These in turn were replaced by small shrivelled

granular clumps scarcely recognizable as leptospirae. Finally all that remained were small granules floating about in the medium. At the highest dose level of 1,000,000 rad thinning of the culture and degeneration of the leptospirae were evident an hour after irradiation but the whole process, terminating in complete death of the culture, required up to 48 hours. Cultures exposed to lower doses of gamma radiation survived up to five days. Doses up to 200,000 rad failed to kill the cultures. When unirradiated L. pomona were inoculated into irradiated media, growth occurred in all tubes indicating that the death of the irradiated leptospira was not due to chemical change in the media.

It was concluded from these observations that the degeneration of leptospirae did not resemble the rapid and complete disappearance observed in the agglutination-lysis test. The explanation of the changes in the organisms submitted to irradiation or in cultures undergoing aging may be explained if one accepts the assumption that leptospirae divide by transverse fission. In an aging culture increasingly large numbers of organisms are found which have a beaded appearance. Finally only granules are seen. These may represent segments of the organism in the reproductive process by transverse fission which has been halted at the segmental stage of exhaustion of nutrients in the media. A similar chain of events seems to appear in cultures submitted to irradiation with the exception that the segmentation is blocked by an alteration of the reproductive ability rather than lack of nutrient.

If, as the evidence so far presented suggests, lysis does not occur in the agglutination-lysis test the organisms must remain somewhere

in the tube undetected by the usual technique of examining a loopful of fluid. A possibility in this regard was that of their agglutination and sedimentation to the bottom of the tube.

Three sera known to be positive and one known to be negative together with a saline antigen control were set up in the agglutination-lysis test in the usual way. The tests were performed in duplicate. Routine examination of serum 1 showed 4+ reactions in the 1:10, 1:100 and 1:1000 dilutions and a 3+ reaction in the 1:10,000 dilution. Serum 2 showed 4+ reactions in the first two tubes and a 2+ reaction in the third. Serum 3 revealed similar reactions to serum 1. Serum 4 and the saline control showed no reaction.

After the routine reading, set 1 was examined by removing a drop from the bottom of each tube by means of a Pasteur pipette. Examination of serum 1 revealed small ropey tangles in the 1:10 dilution and small grape-like clusters of compact balls of agglutinated leptospirae in the 1:100 to 1:10,000 dilutions. No agglutination was found in succeeding dilutions. Findings with serum 2 resembled those of serum 1 in that loose ropey agglutination was found in the first dilution and clusters of lysis balls in the second dilution. Only a few single lysis balls were found in the third tube and the remainder were negative. Serum 3 resembled serum 1 with the exception that in the 1:100 dilution agglutination tended to result in large solid masses rather than in clusters of small balls.

The second set of tests was centrifuged for 20 min. at 2500 r.p.m. in an International Centrifuge Model S.B.V. A drop from the bottom of each tube was examined as before. Results obtained were similar to those

found in set 1 but agglutinated masses tended to be larger and more numerous. For comparison the results of the various examinations are listed in Table XXX. Plates 9 to 14 illustrate the findings in sediment from tubes which gave positive reactions or apparent lysis during routine reading of the tests.

It would be impossible to state that none of the leptospirae had lysed. However, their disappearance in the positive dilutions of the test could easily be accounted for by agglutination and settling in the tube so that they are not picked up by the wire loop. In view of Lawrence's studies which indicated that individual leptospirae are not lysed by antiserum and of the observations described here, it is concluded that lysis in the agglutination-lysis test is apparent rather than real and depends upon the method of sampling.

During the course of these studies it was of interest to note that agglutination in the 1:10 dilution of positive sera differed from that in higher dilutions. In the low serum dilution containing more antibody the organisms tended to agglutinate into ropey structures which formed loosely tangled masses as pictured in plate 9. In higher serum dilutions compact balls and aggregations of these balls were formed. The phenomenon appears to be an example of "prozone" effect in which excess antibody produces a loose type of agglutination with the antigen.

TABLE XXX

APPEARANCE OF THE SEDIMENT FROM AN AGGLUTINATION-LYSIS TEST

Test	Serum dilution 1:10	1:100	1:1000	1:10,000	1:100,000 and 1:1,000,000
Serum 1 Routine A-L	4	4	4	3	-
Sediment	Loose ropey tangles	Small clusters of agglutination balls	Small clusters of agglutination balls	Small clusters of agglutination balls	No aggl.
Centrifuged sediment	Large ropey tangles of leptospirae	Large clusters of small agglut-ination balls	Large clusters of small agglut-ination balls	Large clusters of small agglut-ination balls	No aggl.
Serum 2 Routine A-L	4	4	2	-	-
Sediment	Few clusters and ropey tangles	Several large and many small balls	A few small balls	No aggl.	No aggl.
Centrifuged sediment	Much ropey agglutination a few balls	Several massive balls few small balls	Few small balls and stellate clumps	No aggl.	No aggl.

TABLE XXX.- APPEARANCE OF THE SEDIMENT FROM AN AGGLUTINATION-LYSIS TEST (continued)

Test	Serum dilution 1:10	1:100	1:1000	1:10,000	1:100,000 and 1:1,000,000
Serum 3 Routine A-L	4	4	4	3	-
Sediment	Numerous small ropey tangles	Large dense agglutinated masses	Some large masses and small clusters	Small clusters and single balls	No aggl.
Centrifuged sediment	Large loose ropey tangles	Large dense masses and clusters of small balls	Large clusters of small agglutination balls	Large clusters of small balls	No aggl.
Serum 4 Routine A-L	-	-	-	-	-
Sediment	No aggl.	No aggl.	No aggl.	No aggl.	No aggl.
Centrifuged sediment	No aggl.	No aggl.	No aggl.	No aggl.	No aggl.
Saline A-L	-				
Sediment	No aggl.				
Centrifuged sediment	No aggl.				

DISCUSSION

The remarkable infectivity of L. pomona for young guinea pigs was indicated by the fact that approximately 13 leptospirae representing a dilution of 1:9,480,000 of the original culture produced disease in three of four animals. When approximately seven leptospirae were injected, two out of four animals became infected. It is recognized that the accuracy of such high dilutions is questionable and that some animals may have received no organisms in the inoculum while others may have received several times the calculated number. However, the dilution of infective material was very great and the fact that infection was established in the guinea pigs indicated the high degree of susceptibility of this animal species.

When the number of leptospirae in the inocula was adjusted in proportion to the body weight of each animal species in relation to that of the guinea pig, it was found possible to infect hamsters, rabbits and calves but not swine with a corresponding weight multiple of the guinea pig infective dose. The natural resistance of swine to infection with very large inocula was unexpected as these animals are generally considered to be reservoirs for L. pomona infection. It is possible that subcutaneous injection of the infective material may not have been the best route for challenge but was necessary for accurate dosage and uniformity of technique. While passage of leptospirae through the broken or macerated skin is a recognized natural route of infection in man and has been used experimentally in laboratory animals the more natural route in swine may be the occular,

intranasal or oral ones.

It is interesting to note that swine, which have been shown to be naturally resistant to leptospiral infection, are known to develop chronic foci of infection in their kidneys and to shed leptospirae in their urine for many months. On the other hand, calves are easily infected but shed leptospirae in their urine for only a short time.

Studies of infectivity of L. pomona for guinea pigs have also indicatedthat inocula containing larger numbers of leptospirae than the minimum infective dose do not produce more severe disease. On the contrary, the temperature response occurred earlier when more organisms were injected but tended to be lower. This probably reflects more prompt activation of antibody production under the influence of the larger injection of antigenic material. Ultimate agglutination-lysis titres were not influenced by the numbers of leptospirae in the inoculum.

Immunological studies in guinea pigs using several killed suspensions of leptospirae as antigens showed that some preparations stimulated more protection than others. One antigen appeared to favor infection in that guinea pigs inoculated with it developed leptospirosis in a shorter interval following challenge than non-immunized control animals. Serum agglutination-lysis titres of animals whose immunity was insufficient to withstand challenge tended to be higher than those of infected control animals.

The studies into the immunological reactivity of two of the antigens investigated in guinea pigs were continued in calves. These seemed at first to contradict the results obtained in the guinea pigs

where one antigen gave good protection and the other little or none. It was shown that some replication of leptospirae had occurred in the immunized calves following injection of challenge material but no clinical manifestations of leptospirosis were observed. Only a difference of one dilution in the agglutination-lysis titres was obtained between the groups of calves. Such a difference is considered within the range of technical error and both antigens were thought to have protected the calves to approximately the same degree. However, when the complement-fixation test was employed the difference between the serum titres of the two groups was marked. Those calves injected with the "good" antigen failed to develop complement-fixing antibodies while those which received the "poor" antigen developed high titres. This was interpreted as meaning that the former group had withstood challenge while the latter had developed infection. One calf of this group became a urinary shedder of leptospirae. These tests in guinea pigs and in calves produced essentially similar results and suggest that guinea pigs are satisfactory animals for use in assessing the protective value of leptospiral antigenic agents for cattle. It was also indicated that the complement-fixation test is a better means of determining the status of animals with respect to active leptospiral infection than the agglutination-lysis test.

Water-soluble and water-insoluble fractions of alcoholic extracts of leptospiral cells were without value as immunizing agents. Rothstein and Hiatt found the former to be a precipitinogen and the latter to be a complement-fixing antigen and both were thought to be composed largely of polysaccharides. The complement-fixing antigen used throughout these studies was prepared according to the above authors' technique with

satisfactory results. It would appear that, as with the carbohydrate-like components of other bacteria, these fractions behave as partial antigens or haptenes.

Of the tests employed for the demonstration of leptospirae in tissues and body fluids cultural techniques proved to be most reliable when material was not contaminated with other organisms. Dark-field examination of blood specimens during the acute phase of leptospirosis was moderately successful in calves but almost completely unsuccessful in guinea pigs. Dark-field examination of urine from swine and calves was most satisfactory when conducted at intervals commencing at least two weeks after the acute phase of infection. Fluorescein-labelled antibody techniques could be relied upon only when large numbers of organisms were present in the material being examined and did not appear to offer an advantage over the other techniques.

Of the tests employed for the demonstration of serum antibodies the agglutination-lysis test was the most sensitive indicator of an animal's "experience" with a specific leptospiral serotype. Moderately high titres persist for months after recovery and such titres simply indicate previous contact. Reference has already been made to the possible use of the complement-fixation test as an indicator of the actual status of an animal with respect to active leptospiral infection. The plate-agglutination test results paralleled those of the complement-fixation test more closely than those of the agglutination-lysis test.

Low grade reactions in the agglutination-lysis test were observed in kidney suspensions from infected animals. These were not of sufficient

magnitude to prove renal production of antibody and could have been the result of residual blood in the tissues.

Complement is not required in the agglutination-lysis reaction as it is in bacteriolysis in general. The reason for this is probably that the reaction is not lysis in the true sense of the word, that is complete dissolution of the organism. On the contrary, compact agglutination and possibly some disintegration occurs but the agglutinated clumps have been shown to remain sedimented at the bottom of the tubes where they are overlooked. Lysis is apparent rather than real.

SUMMARY

The susceptibility of guinea pigs to L. pomona infection has been determined with observations regarding the severity of infection and antibody response at various dose levels.

The relative susceptibility to L. pomona infection of various other animal species such as hamsters, rabbits, calves and swine has been studied using multiples of the guinea pig infective dose according to weight.

The immunological activity of seven killed suspensions of L. pomona was compared in guinea pigs. Two of these antigens, the best and the poorest, were also investigated in calves and results were correlated with those obtained in guinea pigs.

Fractions of L. pomona organisms were studied for in vivo and in vitro antigenicity.

Comparisons were made of several techniques such as animal inoculation, culture methods, dark-field microscopy and the fluorescein-labelled antibody technique for the demonstration of leptospirae in infected tissues and fluids.

Serological tests such as agglutination-lysis (microscopic agglutination), complement-fixation and plate-agglutination were also studied. The usefulness of these reactions as indicators of actual infection or subsequent immunity is discussed.

Kidney tissue was considered as a possible site for leptospiral antibody production.

The mechanism of the agglutination-lysis reaction has been investigated particularly with respect to the necessity for complement and the phenomenon of lysis.

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Plate 1.- *L. pomona* in pure culture as revealed by fluorescent microscopy. 320 x.

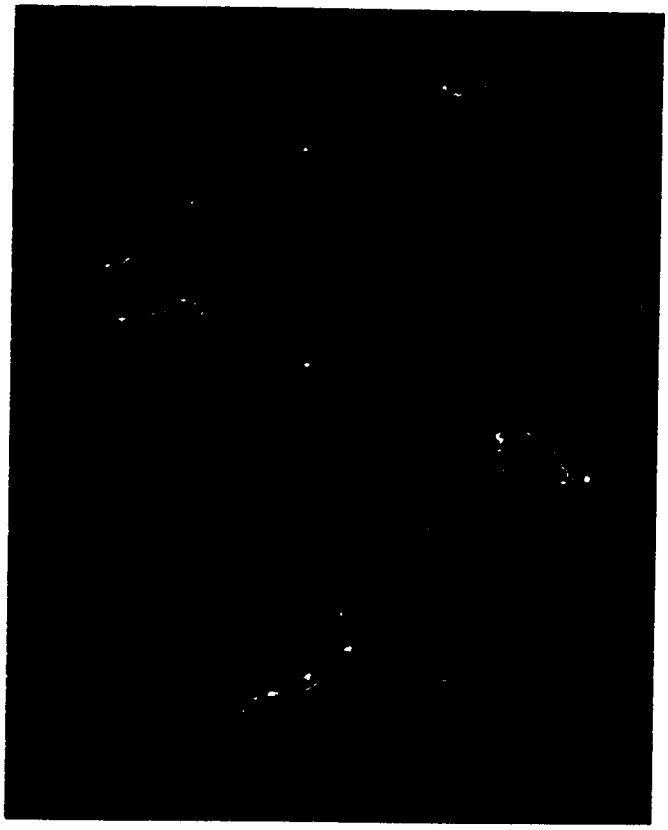

Plate 2.- *L. pomona* in pure culture as revealed by fluorescent microscopy. 800 x.

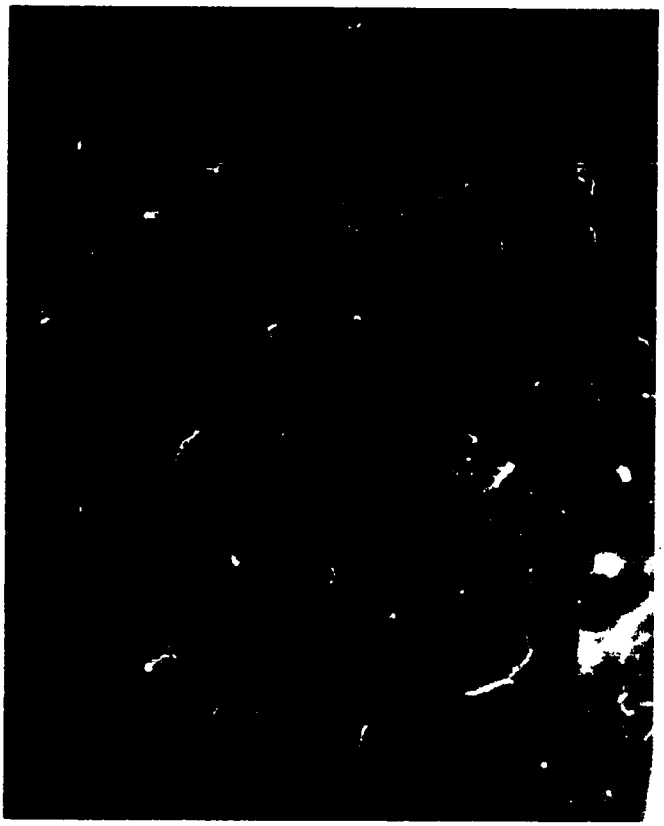

Plate 3.- L. pomona in urine of a calf as revealed by the fluorescent technique. 320 x.

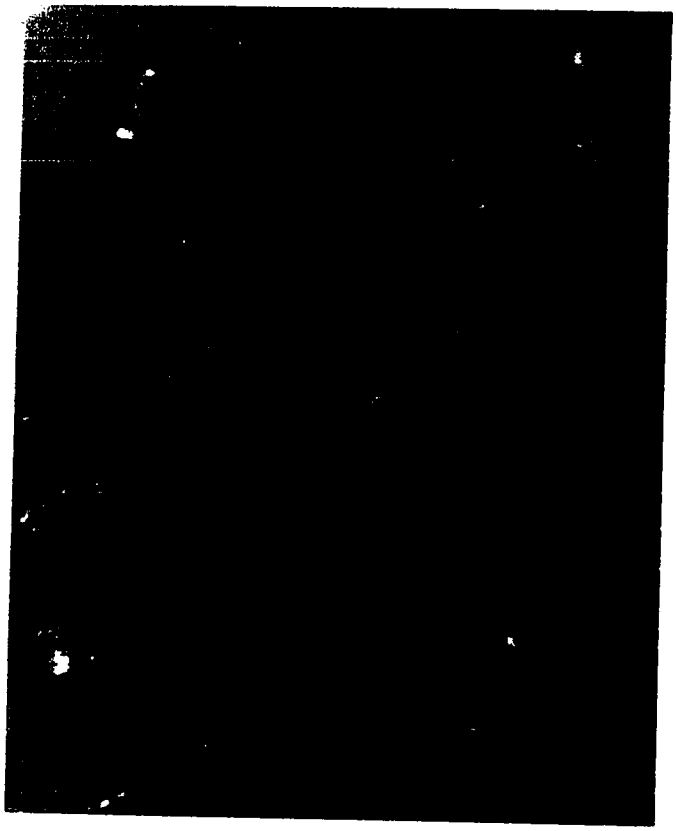

Plate 4.- L. pomona in urine as revealed by fluorescent microscopy. 800 x.

Plate 5.- Leptospirae in calf kidney impressions as shown by the fluorescein labelled antibody technique. 320 x.

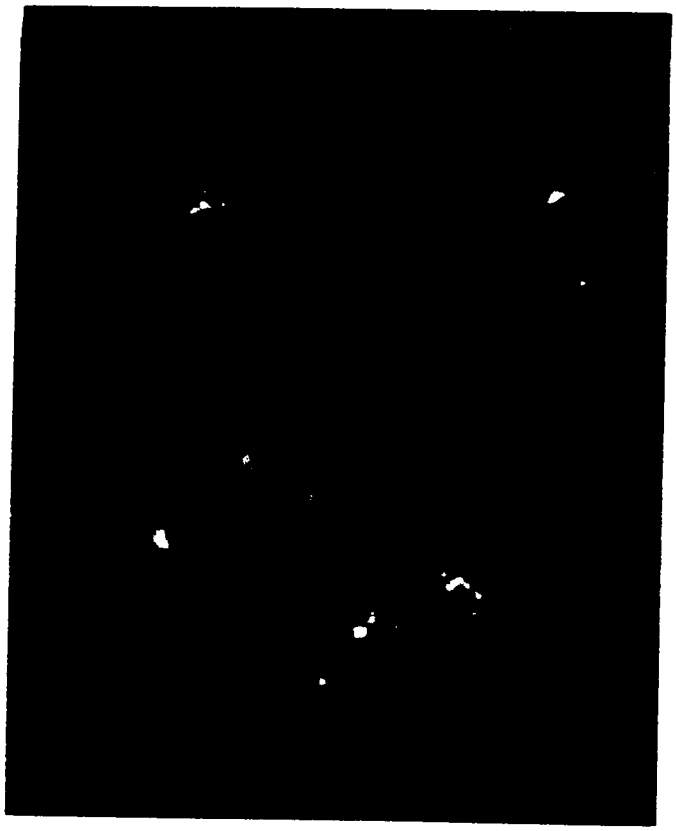

Plate 6.- L. pomona in calf kidney as revealed by fluorescent microscopy. 800 x.

Plate 7.- The kidney of a calf infected with L. pomona showing numerous pale foci scattered over the cortex.

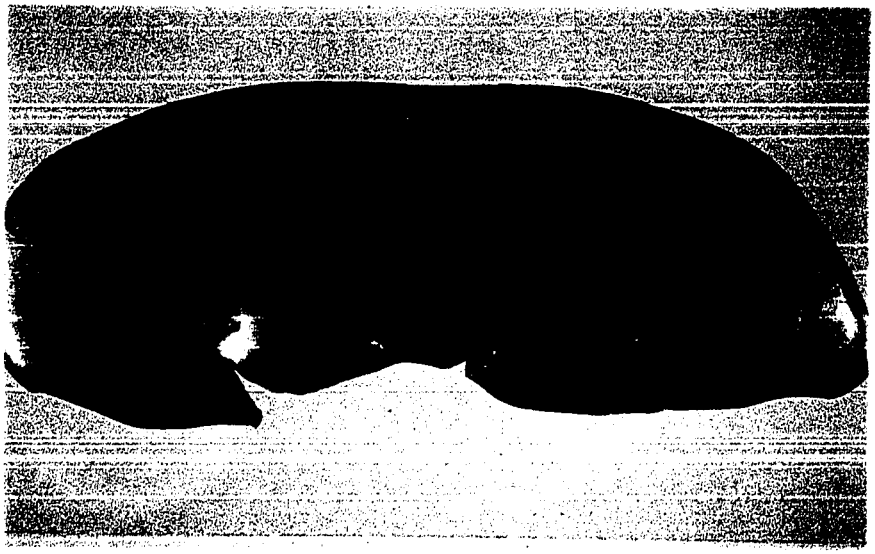

Plate 8.- The kidney of a pig infected with L. pomona showing numerous pale foci scattered over the cortex.

Plate 9.- Loose ropey tangles of agglutination in sediment in high serum concentration showing prozone effect with living leptospirae 500 x.

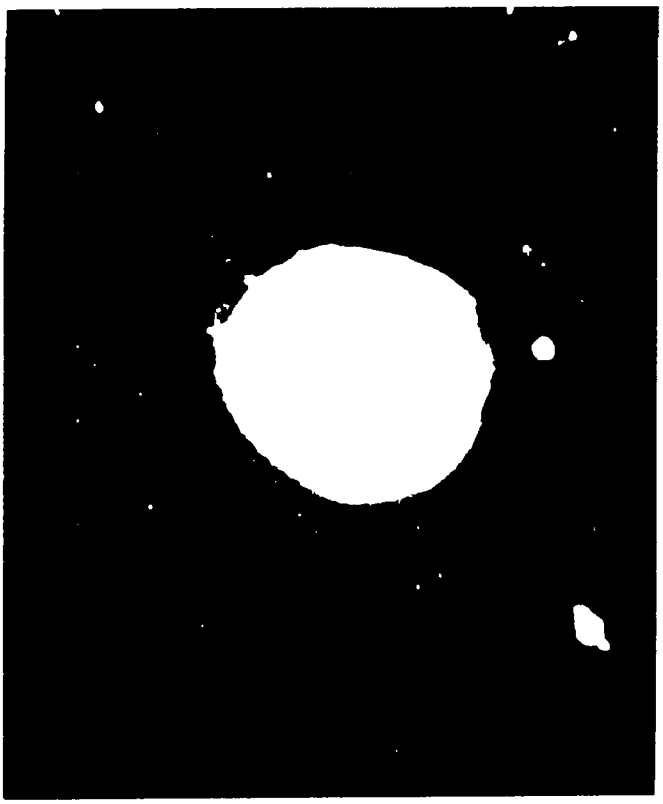

Plate 10. Compact agglutination of living leptospirae forming a "lysis ball". 500 x.

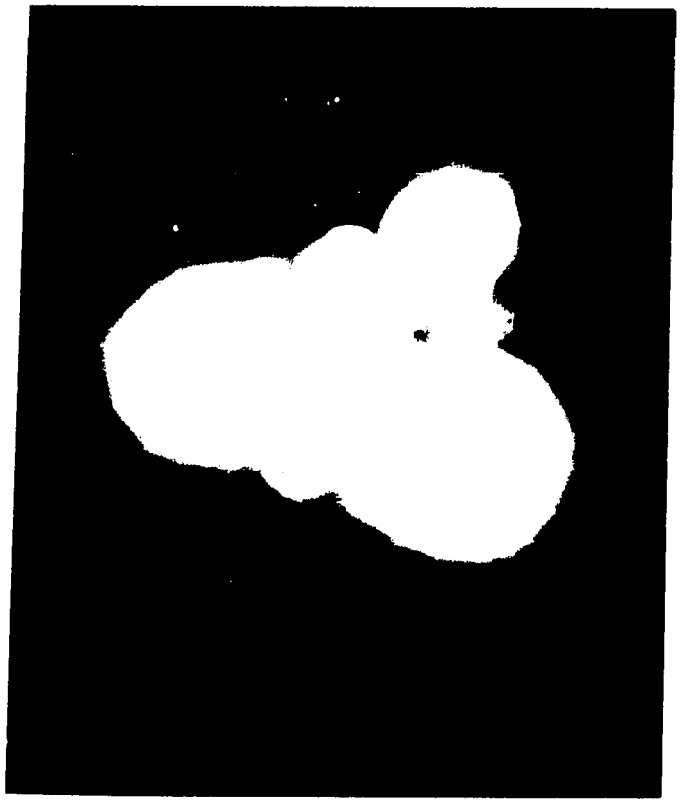

Plate 11.- Several "lysis balls" coalescing to form a larger irregular mass. The fuzzy outline was produced by actively motile organisms protruding from the structure. 500 x.

Plate 12.- Grape-like clusters of small "lysis balls" found in the sediment of most of the positive dilutions. 125 x.

Plate 13.- Clusters of small compact "lysis balls" as viewed by oil immersion microscopy. 800 x.

Plate 14.- A small portion of a very large compact mass of agglutinated leptospirae is seen in the lower left corner of the picture. Several small "balls" are also present. 125 x.