THE TITRATION OF AN IMMUNOGENIC AGENT AGAINST *LEPTOSPIRA POMONA* USING SYRIAN GOLDEN HAMSTERS (*CRICETUS AURATUS*)

by

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II. INTRODUCTION

L. pomona was first isolated in Pomona, Queensland, Australia, in 1936. The case occurred in a dairy farmer who had been suffering from a mild fever for seven days. Several cases similar to this had been noticed in the previous years. The disease in man is known as "swineherds disease" since it was very prevalent among people working around pig sties, the causative agent being isolated from pigs in 1940 (1).

Bovine Leptospirosis was first described in Russia in 1935. Since then, it has been described on all of the continents (2). It was first described in the United States by Baker et al (3), who observed cows whose milk had a bloody or thickened yellow appearance. The affected cows showed fever and occasional hemoglobinuria. When the milk was injected into guinea pigs, rabbits, mice, and embryonated eggs, a febrile response was demonstrated in the rabbits and guinea pigs. The agent which had been in these, when reinoculated into cattle, the resulting injection resembled the natural disease. The organism was

finally isolated and found to be a spirochete resembling leptospira with its hooked ends.

Gochenour et al (4) demonstrated that strains of bovine leptospira of American origin designated as New Jersey C164 and New York A to be indistinguishable from L. pomona by cross agglutination-lysis. In cross adsorption tests L. pomona would absorb all of the antibodies of these two strains, indicating the same species. In animal protection tests, hamsters immunized with strain New Jersey C164 or L. pomona immune sera were protected against infection when inoculated with strain New York A, again indicating these strains to be L. pomona since none of the other species used was protected.

The use of an immunogenic agent in calves has recently been reported by Hoag and Bell (5). Their work shows immunogenic potency differences in various lots of vaccine used in different groups of cattle. The necessity for titration using inexpensive and feasible methods in the laboratory before inoculation of the vaccine in cattle is evident (6).

The use of effective chemotherapy and antibiotic therapy has not taken great strides toward the cure

of leptospiroses. This is particularly true in man although some limited success has been attained in small laboratory animals when the disease was experimentally induced (7). However, in a natural case of the disease diagnosis is not usually made until the disease is well advanced and damage already done.

Laboratory diagnosis has been hampered by limited facilities and trained personnel. Lack of knowledge of the organism itself other than its morphology has slowed down advances in diagnosis, prophylaxis, and therapy (8). Induced immunity seems to be the logical solution to the problem, and with these thoughts in mind the following project was proposed: to develop an evaluation technique using weanling golden Syrian hamsters for the purpose of titrating the immunogenic potency of a leptospiral vaccine.

III. REVIEW OF THE LITERATURE

A. Classification

Broadly, the leptospira have been classified in relationship to other bacteria by their typical appearance, their locomotive and pathogenic characteristics. Schuffner (9) as quoted by Van Theil (10) made the following classification:

Genus Spirochaeta

Subgenus Borrelia

Subgenus Treponema

Subgenus Leptospira

The various species or strains of leptospira cannot be differentiated morphologically, or biochemically, but only by means of serological tests. The tests which are commonly used are microscopic agglutination-lysis test, macroscopic agglutination tests, and complement fixation tests.

Gochenour et al (11) used a group of 16 serotypes as a screening device for the approximately 26 known serotypes, using the cross agglutination-

lysis method. This has helped lead to the discovery of previously unreported types. The process is one of agglutination followed by that of lysis, the lytic process being able to take place without the presence of complement as is needed with other bacteria (10).

Hoag et al (12) devised a means whereby classification of the leptospira could be made using killed leptospira in a macroscopic agglutination test. Antigens were tested for optimalcy with respect to density, pH, and ionic strength. Using a dye for ease of discernibility, the test proved comparable to the microscopic method with the expection of low titer sera.

Complement-fixation tests were carried out essentially the same as any other test. A method using soluble antigens will be discussed later.

B. Nutrition of Leptospirae

Very little is known about the nutritional requirements of the leptospirae other than that the pathogenic types require mammalian serum, egg fluids, or similar complex substrates. The yield of organisms is low in proportion to the amount of serum protein used which has slowed down the progress of chemical investigation (13).

Chang (14) made a series of physical growth tests and nutritional requirements. He concluded that the growth pattern was similar to that of other bacteria but much slower. (Mycobacterium tuberculosis, one of the slowest growing bacteria, grows five times faster.) He found the optimum temperature to be about 28 °C. and grew best at a pH near 7.2.

Chang found serum to be essential for growth in cultures and that growth supporting factors in serum were destroyed by heating at 100 °C. for less than one minute. He tried adding vitamins, glycerol, cytochrome C and crystalline serum albumin, but these growth factors could not be entirely replaced for whole serum since the culture would die out after two to three transfers. Sugars were also tested,

as well as glucose and other hexoses, and quantitatively were found not to be used.

As to the oxygen requirement, Chang found them to be obligate acrobes growing most optimally when oxygen was abundantly supplied. Czekalowski et al (15), however, tend to discredit this by inferences to the Dinger phenomenon. (This is a condition in which the cultures in a solid or semi-solid media develop into thick masses in the form of rings a few millimeters below the surface, the under surface of the ring being well defined.) It was surmised that these rings grew at a level of optimum oxygen tension and might even be called micro-aerophilic. When other organisms were tested for this phenomenon, the leptospirae had an appearance which appeared to be characteristic.

In a later paper, Czekalowski et al (16) used the speed of development of these rings as a criterion of the nutritive value of media. Using peptones made by different companies, they concluded most peptones in suitable concentrations will increase the speed of growth in a basal medium of 10% (V/V) rabbit serum in diluted meat extract. At concentrations

above 0.1%, however, some peptones had an inhibitory effect. This was particularly so with autoclaved peptones in slightly alkaline solutions as opposed to filtered peptones.

Czekalowski (15) demonstrated that the leptospirae had no catatase so that hydrogen peroxide would be able to accumulate. Bedford (17) believes that the destruction of microorganisms is proportional to the production of hydrogen peroxide. Hydrogen peroxide can be produced by the ultra-violet rays of light. This could be an argument for the inclusion of laked hemoglobin (rich in catalase) into the media. However, Wiesmann (18) [as stated by Czekalowski (15), and Chang (14)], found no advantage in using the laked hemoglobin, presumably the fresh serum containing enough.

C. Antigens

In immunochemical studies on the leptospirae conducted by Schneider [as reported by Hiat (19)], the following was found. After disrupting the cell by an appropriate means, and extraction with a mixture of amyl alcohol and chloroform, the aqueous phase was concentrated. The resultant liquid contained about 40% of the total solids of the organisms. It reacts positively in the Molisch test and negatively in the biuret and ninhydrin test. This indicates the product is essentially free of proteins, pepides, and amino acids but indicates the presence of carbohydrates. This fraction was found to contain a thermostable complement-fixing antigen that is serotype specific and reacts with heterologus leptospiral antisera. However, antisera produced against this fraction does not have the ability to agglutinate living leptospires, although this does not interfere with the agglutination-lysis reaction, indicating that there might be more than one specific antigen system in the leptospirae.

Ezell et al (20) reported a soluble antigen which was highly strain specific for leptospirae. Organisms which were artifically grown were centrifuged and the supernatant liquid filtered through a Seitz filter. The centrifuged organisms were resuspended and subjected to high velocity vibrations and sonic vibrated type antigens prepared. The filtrate (containing the soluble antigens) when compared to a whole leptospiral culture in a complement fixation test using hyperimmune sera gave the same result, i.e., 1:320. When compared to the sonic vibrated antigen (minus the soluble antigen) the result was only 1:40. In cross complement fixation tests the soluble antigen is highly strain specific.

Hoag et al (21) reported a soluble antigen prepared by the acid heat extraction of living cultures of Leptospira pomona for use as an immunogenic agent. The findings were that there was no correlation between the amount of agglutinating antibodies and immunity. Calves which were well protected by this soluble immunizing agent produced little or no agglutination-lysis antibody. This fact could be used to advantage since the laboratory diagnosis of

leptospirosis usually involves the detection of agglutinating antibodies. It would make less confusing the diagnosis of artifically immunized, naturally immunized, and infected animals.

Usually, in testing for the immunogenic potency of a vaccine there is a delay of approximately ten days before there is a maximal amount of circulating antibodies and before the experimental animal is challenged. Larson et al (22) were able to demonstrate in mice some resistance to challenge within 24 hours after inoculation with soluble antigens and a maximal development by the third day. However, agglutinins could not be demonstrated in animals immunized with soluble antigens.

D. Adjuvants

It was noted that tubercular guinea pigs when injected with some other antigen, gave a greater antibody response than nontubercular guinea pigs. It followed that an antigen combined with dead Tubercule bacilli might have the same effect. Using a lanolin-like substance and dead Tubercule bacilli suspended in paraffin oil as the adjuvant, this proved to be true (23).

In an experiment with typhoid bacilli, Freund et al (24) found that the adjuvant (this time with liquid petrolatum instead of paraffin oil) not only augmented antibody production but greatly lengthened its duration. "The effect of the adjuvants studied might be due to the protection of the antigen against destruction and elimination."

It is to be concluded that any antigen is augmented by an adjuvant.

E. Why Use Hamsters?

In an experiment in which the evaluation of a vaccine is to be made, it is not enough that the test animal become vaguely ill with an occasional death but in a fair proportion of the time, death must occur. Most of the work done for the selection of a suitable laboratory test animal has been done with Leptospira icterohemorragiae (25) (26) (27) (28).

Packchanian (25) studied the susceptibility of 32 species and subspecies of rodents to virulent strains of L. icterohemorragiae and concluded that certain species of American deer mice genus Peromyscus are suitable as susceptible small laboratory animals. However, no hamsters were used.

Larson (26) found that young white mice Mus musculas are extremely susceptible to L. icterohemorragiae with mortality rates approximating 100% in three-week old mice. Packchanian (25) found these white mice to be completely unsusceptible. Morton (27) found that hamsters so inoculated succumbed in five to eight days.

In a later paper, Larson (28) showed hamsters to be susceptible to both L. ichterohemorragiae and L. canicola, and Randall (29) made the same observations, together with the fact that young guinea pigs and mice were resistant to infection with L. canicola and rats entirely so. Gochenour et al (30) found greater success was obtained in leptospirosis when using hamsters than when using guinea pigs. The hamster seems to be the test animal of choice.

F. Route of Inoculation

There are indications that the route of inoculation of an infectious agent has some bearing on the LD_{50} of a test animal, whether it be immunized or unimmunized. Bell et al (31) demonstrated that the protection afforded immunized rats against intraperitoneal challenge was slight, whereas the protection against subcutaneous challenge was relatively high. This has been found to be true by other investigators (32) (33) but varied somewhat with the species. Larson (34) found that with hamsters, there appeared to be an equally susceptible condition whether the challenge was administered subcutaneously or intraperitoneally.

The efficacy of the vaccine itself appeared to be little influenced by the route of administration (31) (35).

IV. METHODS AND MATERIALS

A. Preparation of the Immunogenic Agent

Leptospira pomona 285, was inoculated into Stuart's liquid media (36) in 500 ml, screw-capped bottles for 10 days at 30 °C., that being the time it took for the growth to become heavy. The serum used in Stuart's media was prepared from whole, fresh rabbit blood. The blood was centrifuged in an International Refrigerated Centrifuge for 15 minutes at 20,000 revolutions per minute and at 10 °C. temperature. The serum was sterilized by twice passing it through a Seitz filter with a serum #1 pad and then inactivated for 40 minutes at 56 °C.

After a heavy growth of the organisms had been obtained on Stuart's medium the HB vaccine (21) was made from it. The volume of pooled culture material was measured and one-tenth normal acetic acid was added in an amount equal to 3 per cent of the total volume. The acidified pooled material was heated to 100 °C. for 10 to 15 minutes, or until a heavy, white precipitate formed. After cooling to room temperature,

the precipitate was filtered out and resuspended in a small amount of distilled water. Enough one-tenth normal NaOH was added to cause complete solution of the resuspended precipitate. This resuspended material was then diluted to one-fifth of its original volume with distilled water and preserved with merthiolate at a final concentration of 1:10,000. Two parts of this final concentration were added to three parts of an adjuvant composed of two parts liquid petrolatum and one part lanolin. The components were well mixed and stored in a refrigerator until ready for use.

B. Preparation of the Challenge Material

The challenge material consisted of heart's blood from Leptospira pomona 285 infected, moribund hamsters. During the whole of the experiment it was necessary to keep a viable source of L. pomona by transferring the blood serially every three or four days from one group of hamsters to another. At each transfer a drop of the blood was examined under oil immersion (97 x objective and 10 x occular) of the dark field microscope for an immediate

confirmation of the presence of leptospira. However, it was not always possible to be positive of the presence of the organisms in the blood smear. It was necessary to corroborate this by inoculating test tubes containing either Flecher's semi solid media or Schuffner's media (37) with the questionable blood. Each of these media was dispensed in 5 ml quantities in screw-capped 16 mm x 125 ml test tubes.

C. The Test Animals

All of the test animals came from the same stock farm and were two weeks old upon arrival at the laboratory. Enough animals for the entire experiment were obtained at one time. Observations were made for only a three day period since young hamsters were more susceptible to infection.

The entire lot of hamsters for one complete experiment was vaccinated on the same day. Five hamsters from a group were put in a glass jar; the bottom of the jar was covered with wood shavings and the top with a wire mesh lid. The jars were placed on wooden shelves in a temperature controlled

room at 72 °F. The animals were fed rabbit pellets and watered ad libitum. Deaths were checked twice daily and the dead hamsters immediately removed from the jars.

D. Immunization of the Hamsters

The immunizing agent, which had been stored in the refrigerator, was warmed until it became liquid. A single dose of HB vaccine was injected subcutaneously into the nape of the neck of 15 to 20 gram weanling hamsters, the amount given depending on the ensuing protocol. Two weeks after the vaccination, the challenge material was inoculated intraperitoneally. The challenge material had been diluted to the proper concentration with 30 °C., sterile, isotonic saline in 5 ml vaccine bottles with skirted-diaphragm, rubber stoppers. Five-tenths of the appropriate dilution was injected intraperitoneally per hamster.

The experiment was run twice, the only difference in the second experiment being that an additional 30 hamsters were used since there were

indications from the first experiment that a smaller dose of vaccine should be included.

The final experiment in which an additional 0.05 ml in the series was included, was set up in the following manner, each block representing five hamsters.

		Challenge Dilutions					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
	control						
	0.05 ml						
ml vaccine	0.10 ml						
	0.20 ml						
	0.50 ml						

Two weeks after the challenge had been administered the number of deaths and survivals were tallied. From this the LD_{50} of the challenge (38) (39) and the corresponding protective power of the vaccine was determined (40).

E. Determination of the Titre

Blood from anesthetized survivors was taken by heart puncture and the blood from any one group (e.g., 0.5 ml vaccine, 10^{-1} challenge) was pooled. This pooled blood from any one group was centrifuged for 10 minutes at 20,000 rpm and at 10 °C. The sera was then dispensed into 5 ml screw-capped vials and stored in a -20 °C. freezer until it was convenient to determine the titre.

The titre was determined by the agglutination-lysis technique (41). The sera was diluted with 0.85 per cent NaCl to provide fourfold dilutions from 1:10 through 1:5120. Six-day old cultures of L. pomona were used as antigens. Two-tenths ml of the antigen was added to 0.2 ml of the diluted sera in Kahn tubes. The Kahn tubes were incubated at 30 °C. for three hours and then examined by low dry (10 x objective, 15 x ocular) using the dark field microscope.

F. Determination of LD_{50} and Protective Index

The following is the protocol that was used to determine the lethal dose (LD_{50}) in which one-half of the animals die. This end point is less affected by chance variations than any other and the procedure was worked out statistically for any variables which might be encountered (39). The following is given as an example, using the control with the various dilutions of the challenge material.

Dilution of Challenge Material

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Mortality rate	5/5	5/5	4/5	2/5	1/5	0/5
No. died	5	5	4	2	1	0
No. survived	0	0	1	3	4	5
Accumulation totals						
Died	17	12	7	3	1	0
Survived	0	0	1	4	8	13
Mortality rate	17/17	12/12	7/8	3/7	1/9	0/13
% Mortality	100	100	87	43	11	0

Mortality rates were expressed as a fraction in which the numerator represents the number of hamsters that died and the denominator the number of hamsters at the beginning of the experiment. The number of deaths and survivals is self-explanatory.

In order to get the accumulated total of deaths, the highest dilution containing a death was added to the deaths of the next lower dilution and so on, accumulatively. For example, 10^{-5} dilution of the challenge material killed one hamster and 10^{-4} killed two hamsters, the accumulated total would be three since if 10^{-5} killed one it is assumed it would also have been killed in the 10^{-4} dilution. The same procedure was followed for the accumulated survivals, starting instead at the lowest dilution and adding to the next higher dilution.

In the 10^{-3} group 87% were killed and in the 10^{-4} group 43% were killed, so that the titer was between these two dilutions. It was necessary to determine the proportionate distance between the two dilutions at which the 50% mortality end point occurred, and this was done with the following formula.

$$\frac{\text{\% mortality above 50\% - 50\%}}{\text{\% mortality above 50\% - \% mortality below 50\%}} = \text{factor or proportionate distance}$$

$$\frac{87\% - 50\%}{87 - 43} = 0.8 \quad (0.84)$$

Since the titer was between 10^{-3} and 10^{-4} the proportionate part was 0.8, making the 50% end point $10^{-3.8}$. When tenfold dilutions are used, LD_{50} = log of the titer, or 3.8.

The protective index of the vaccine is the difference between LD_{50} of the control or unvaccinated group and that of the immunized animal (40).

V. RESULTS

On the following pages the results of the two experiments are shown in tabular and graphical form; these include the LD_{50} of challenge and the protective indices of various doses of vaccine.

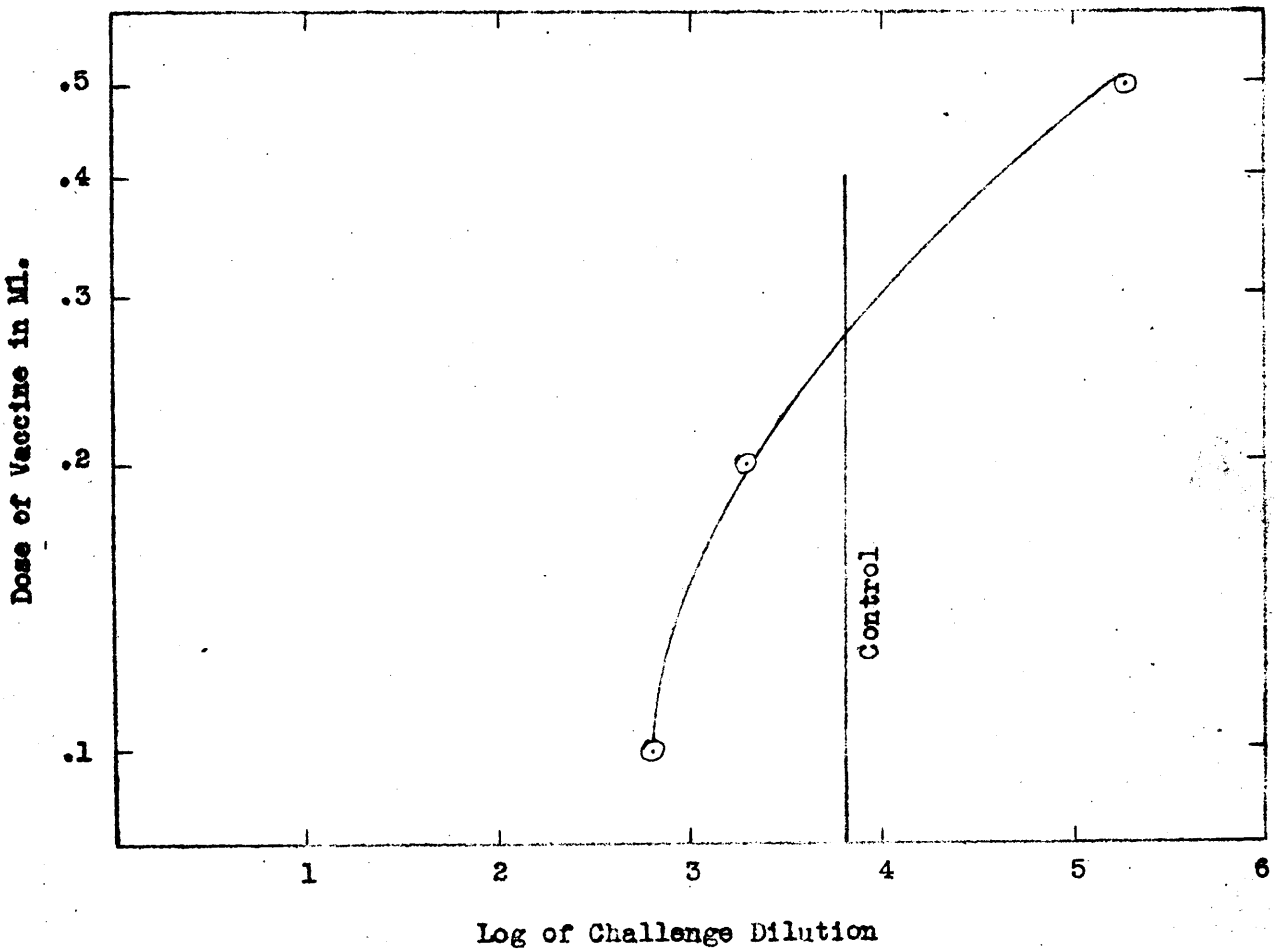
Hamster Mortalities

Experiment 1

Log of Challenge	1	2	3	4	5	6	Pre-Challenge Deaths
Control	5/5	5/5	4/5	2/5	1/5	0/5	0
1.0		4/4	1/4	0/4	0/4	1/5	4
2.0		5/5	3/5	0/5	0/4	1/5	1
5.0		5/5	5/5	2/2	3/5	1/5	3

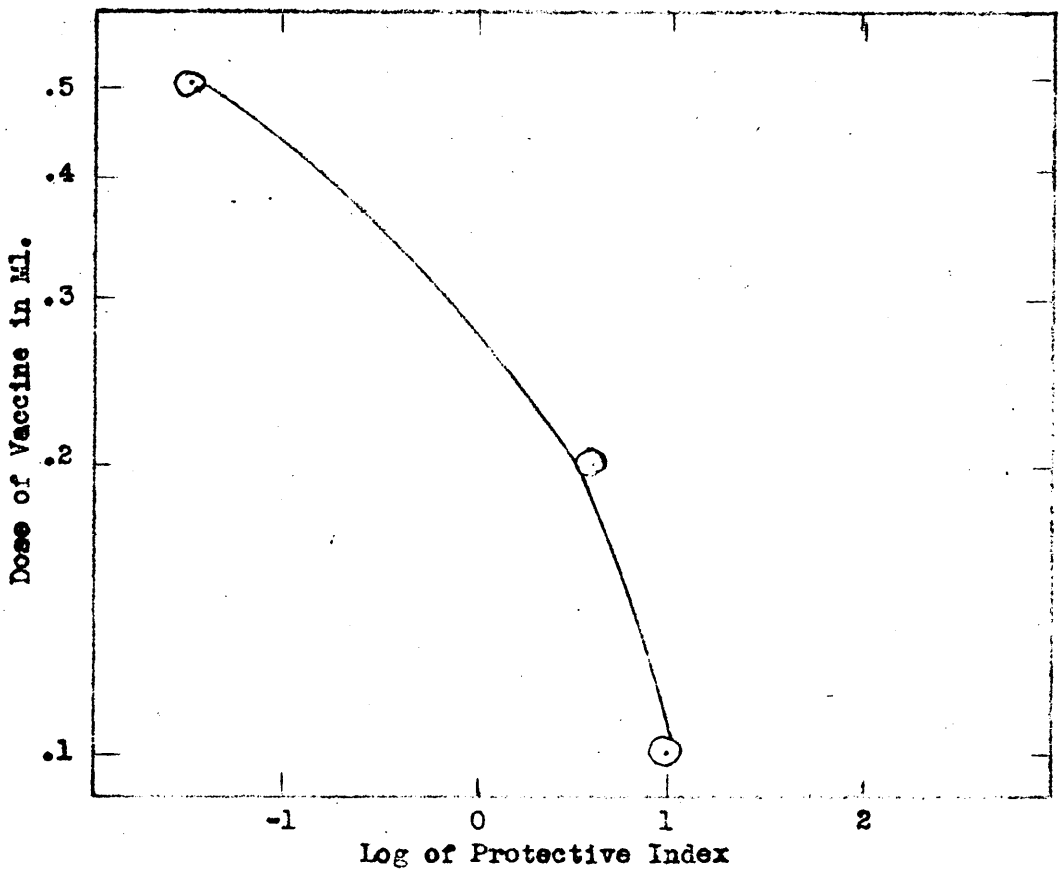
Experiment 2

Control	4/4	4/4	5/5	0/4	0/5	0/5	3
.05	2/5	1/5	0/5	0/4	0/5	0/4	3
0.1	4/5	1/5	2/5	0/4	0/5	0/5	1
0.2	4/5	3/5	2/5	0/5	1/5	0/4	1
0.5	4/5	2/4	3/4	2/4	0/5	0/5	3



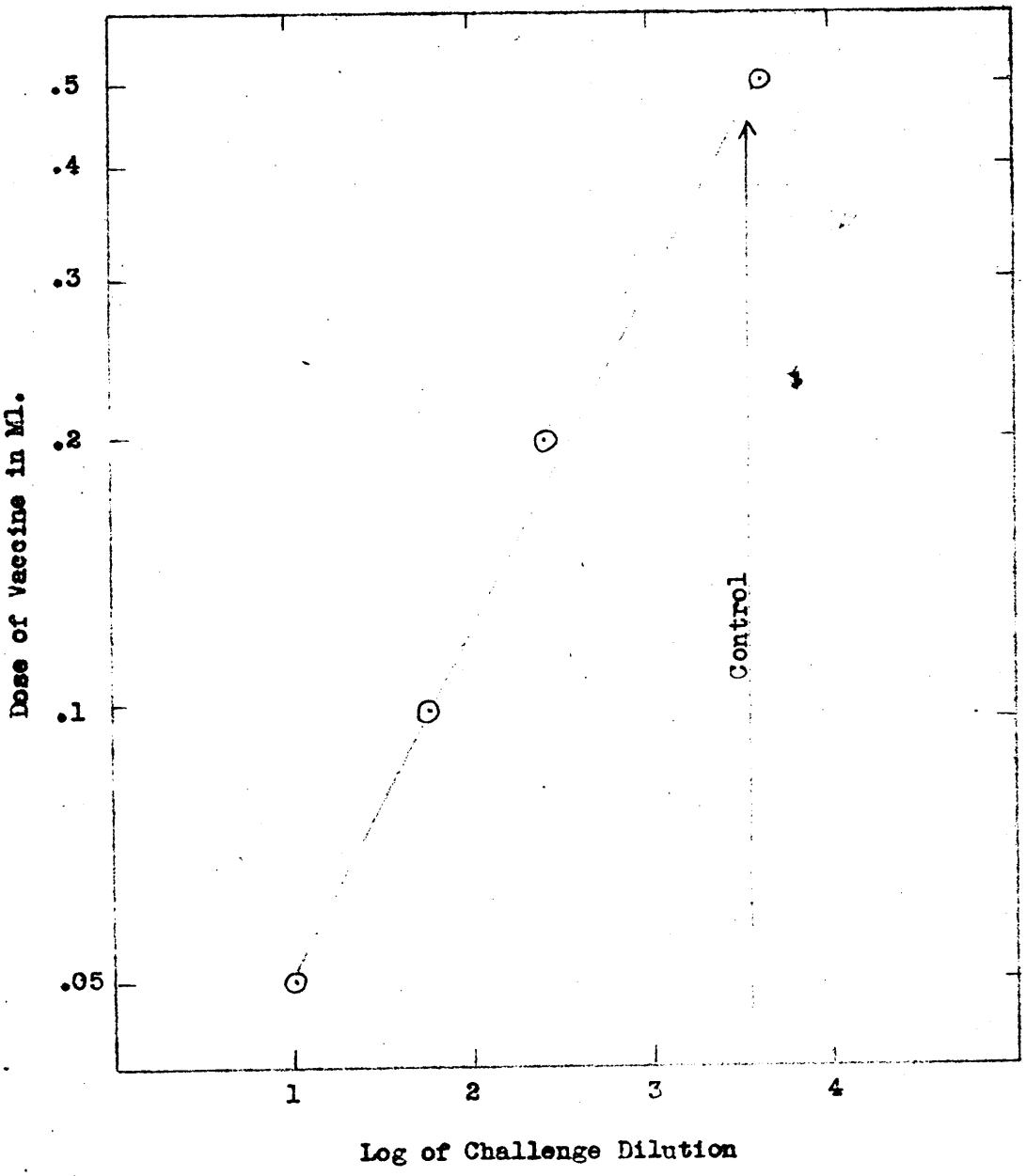
LD_{50} OF CHALLENGE

Figure 1 Experiment 1



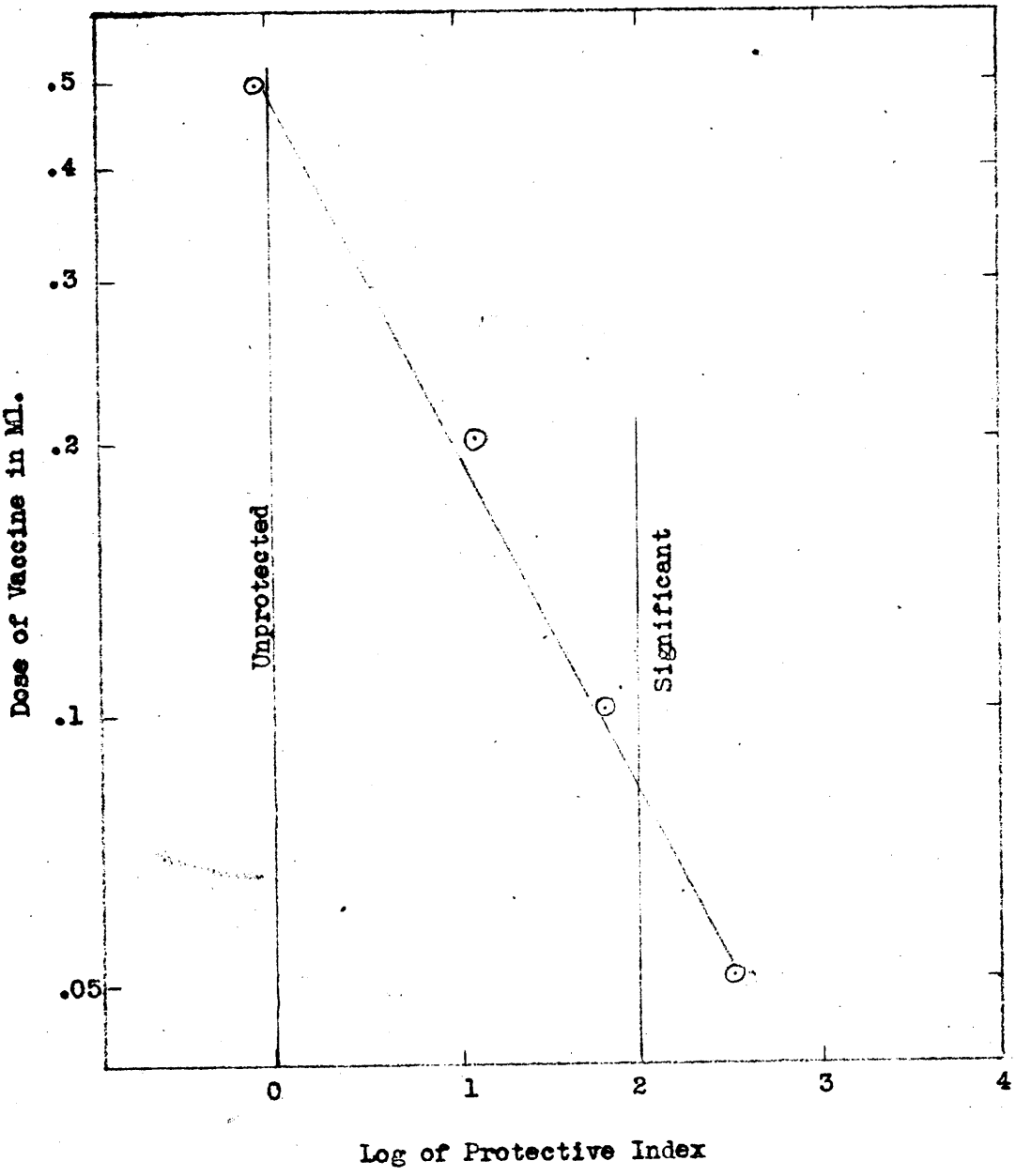
PROTECTIVE INDICES OF VARIOUS DOSES OF VACCINE

Figure 2 Experiment 1



LD_{50} OF CHALLENGE

Figure 3 Experiment 2



PROTECTIVE INDICES OF VARIOUS DOSES OF VACCINE

Figure 4 Experiment 2

VI. DISCUSSION OF RESULTS

The sequence of results was diametrically opposed to what the author had anticipated, it being evident that some form of "blockade" or as Felton (42) calls it, "immunological paralysis" had occurred. It has long been known that the cells of the reticulo-endothelial system act as phagocytes for any foreign particulate matter, this being proven by the use of particulate dyes. It was assumed that any cell which had this power should be concerned either directly or indirectly in the formation of antibodies.

Gay et al (43) saturated the reticulo-endothelial system of rabbits and rats with trypan blue with almost daily injections for two weeks. Serum tested five days later showed the controls to have a titer 75 times greater than the blocked animals.

Tuft (44) made the blockade by means of India ink over a two weeks period followed by inoculations with one of the following: Bacillus typhosus, Bacillus paratyphosus A or B. The agglutinin titers were found to be either absent or negligible as compared to the controls.

The list of experiments made on this subject is large and is by no means all directed in the direction that there even is such a thing as a blockade. Lewis et al (45) albeit found a stimulating effect on antibody activity after an intensive treatment with trypan blue throughout the course of the reaction period in testing for hemolytic amboceptor production.

However, the above particulate dyes were not antigenic substances by nature. Using a dark red dye-portion, R-salt-azo-benzidine-azo-egg albumin, synthesized especially for its positive identification in the cell and for its antigenicity by Heidelberger et al (46) the work of Sabin (47) threw new light on the mechanism of antibody production. She observed that the aggregated, marked antigen after being engulfed by the phagocyte was taken into the vacuole of the cell. There the dye became split from the protein portion and the visible protein aggregates then were apparently made soluble and passed into the cytoplasm of the cell where due to its own innate characteristics caused the synthesis of a specific globulin known as the antibody. Concomittant with the disappearance of the dye and protein particles,

there was a shedding of part of the cytoplasm of the cells and the beginning of the rise of the antibody titer. The implication is that the sloughed off portion contains both antibody globulin and normal globulin both of whose titer or quantity rises at this time.

It would thus seem logical that a paralyzing dose of antigen injures or alters the cells of the defense mechanism so that it is no longer able to function or synthesize antibodies.

It is to be noted from the graphs that in both experiments there was a decided blocking effect when 0.5 ml of the immunogenic agent was used. Even though the "immunological paralysis" was not apparent in any of the other doses of the vaccine, as the doses became progressively smaller in the series the protective power became progressively greater. After the tabulations of the results in experiment 1, it was hoped that a sufficiently small dose had been added to the series for experiment 2 so that there would at least be an increase in protective power from the smallest to the next smallest dose. However, this did not prove true.

The vaccine used in experiment 2 was the same one which was used in the previous experiment, making it approximately three months old. It was thought that by using the same vaccine a more fair comparison might be made between the two experiments, providing of course, that no deleterious effects appeared during the storage period which was at 5 °C. That no deleterious effects appeared seems certain in that the protective index of the vaccine in experiment 2 was greater.

The thought must be kept in mind that the virulence of the challenge might have been different in the two experiments, although the challenge material had been consistently killing hamsters in three or four days prior to their use in the experiments. Another variable in the experiment, assuming the virulence to be the same, was the number of organisms per milliliter of blood, which is quite possible between the two experiments.

Findings of the agglutination-lysis test were inconclusive as to meaning, so they were not tabulated in the section on results. Although the tendency was for the lower dilutions of challenge to give higher

titer values, the results were not consistent as the challenge dilutions became higher. This may be attributed to the fact that the serum from one hamster in some cases was compared with the sera from as many as five hamsters.

VII. SUMMARY

Tests on hamsters reveal that there is a decided protective index from the soluble antigen prepared by the acid-heat extraction of living cultures of Leptospira pomona. They also reveal that the antigen must be given in quantities sufficiently small to be amenable to the system of the test animal or a condition of "immunogenic paralysis" results.

Due to the number of hamsters in any one group whose pooled sera was tested, a fair evaluation could not be made between various groups in the agglutination-lysis test.

Tests on the stability of the immunogenic agent used show that it is completely stable for at least a period of three months when stored in a refrigerator at 5 °C.

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