A SOLUBLE ACID-HEAT EXTRACTED BRUCELLA VACCINE: IMMUNOLOGICAL AND PHYSIOLOGICAL STUDIES IN GUINEA PIGS, RABBITS AND CALVES.

by

Robert Carter Allen, B.S., M.S.

Thesis submitted to the Graduate Faculty of the

Virginia Polytechnic Institute

in candidacy for the degree of

DOCTOR OF PHILOSOPHY

in

Bacteriology

April 21, 1959

Blacksburg, Virginia

TABLE OF CONTENTS

	Page
TABLE OF CONTENTS	2
LIST OF FIGURES	7
LIST OF TABLES	9
I. INTRODUCTION	12
II. REVIEW OF LITERATURE	14
Characteristics of Brucellae	14
Brucellosis in Cattle and Other Domestic Animals	16
Diagnosis	18
Control of Brucellosis in Cattle by the Use of Vaccines	20
Antigens of the Genus Brucella	27
Soluble Extracellular Immunogenic Agents	30
III. OBJECTIVE OF STUDY	32
IV. METHODS AND MATERIALS	33
A. Brucella Cultures	33

Page

B. Media 33

C. *Brucella* Recoveries 34

D. Agglutination Test 35

E. Bacterial Counts 36

F. *Brucella* Challenge Organisms 36

G. Serum Preparation for Serology and Electrophoresis 37

H. Paper Electrophoresis 37

I. Chemical Tests 38

J. Care and Handling of Animals 38

V. EXPERIMENTAL RESULTS 42

A. Selection of an Effective Method for the Preparation of a Soluble *Brucella* Vaccine 42

Experiment I. A Preliminary Study of an Acid-Heat Treated *Brucella abortus* Strain 19 Vaccine in Calves 42

Procedures 42

Results 43

Experiment II. Preliminary Study of an Acid-Heat Extracted 48-hour Growth Culture Vaccine in Guinea Pigs 46

Page

Procedures 46

Results 49

Experiment III. Preliminary Studies on an Acid-Heat Extracted 13-Day Growth Culture vaccine 53

Procedures 53

Results 53

B. Characteristics and Purification of Acid-Heat Extracted Soluble Antigens and their Responses in Guinea Pigs and Calves 59

Experiment IV. A Comparison of Soluble Acid-Heat Extracted Whole Culture and Cell-Free Culture _Brucella_ _abortus_ Vaccines 60

Procedures 60

Results 61

Experiment V. Response Studies of Acid-Heat Extracted Whole Culture and Cell-Free Culture Vaccine in Calves 71

Procedures 71

Results 72

C. Rabbit Experiments: Selection of Normal Animals and the Effect of Sex and Age on the Immunologic Response to Whole Culture _Brucella_ Vaccines 77

		Page
	Experiment VI. Paper Electrophoretic Analysis of Rabbit Serum as an Aid in the Selection of Experimental Rabbits	78
	Procedures	78
	Results	79
	Experiment VII. The Effect of Sex and Age on the Brucella Agglutinin Titer Response and Serum Protein Changes in Rabbits Vaccinated with Whole Culture Vaccines	89
	Procedures	89
	Results	90
VI.	DISCUSSION	109
	A. Immunogenic Studies	109
	B. Rabbit Studies	113
VII.	SUMMARY	117
VIII.	ACKNOWLEDGEMENTS	120
IX.	BIBLIOGRAPHY	122
X.	VITA	136

Page

XI. APPENDICES 139

A. Bacteriological Media Formulas 139

1. *Brucella* Broth 139

2. Host Blood Medium 139

3. Stuart's Medium 140

4. Trypticase Soy Broth 140

B. Animal Feed Formulas 141

1. Rabbit and Guinea Pig Feed 141

2. Calf Developer 142

3. Clovite 143

LIST OF FIGURES

Page

Figure 1 Geometric mean titers and Brucella abortus recoveries 28 days post-challenge from vaccinated and control guinea pigs. Experiment II 51

Figure 2 Geometric mean titers and Brucella abortus recoveries 28 days post-challenge from vaccinated and control guinea pigs. Experiment III 57

Figure 3 Growth response studies of Brucella abortus Strain 2308 grown in Stuart's medium containing 10 per cent rabbit serum 62

Figure 4 Paper electrophoretic patterns of whole and cell-free culture acid-heat extracted Brucella abortus vaccines prepared at intervals during the 13-day growth period 65

Figure 5 Geometric mean titers and Brucella abortus recoveries 28 days post-challenge from vaccinated and control guinea pigs. Experiment IV 69

Figure 6 Temperature and Brucella abortus recoveries following challenge of non-vaccinated and vaccinated calves receiving 13-day whole and cell-free culture acid-heat extracted vaccines. 74

Figure 7 Paper electrophoretic serum protein patterns of normal and abnormal rabbits 80

Figure 8 Agglutinin titer decline in male and female rabbits one-year-old vaccinated with approximately 20 mg protein of Lot 3 vaccine without adjuvant 95

Page

Figure 9 Agglutinin titer decline in male and female rabbits six-months-old vaccinated with 20 mg protein of Lot 4 vaccine without adjuvant 97

Figure 10 Agglutinin titer decline in male and female rabbits one-year-old vaccinated with 30 mg protein of Lot 7 vaccine without adjuvant 99

Figure 11 Agglutinin titer decline in male and female rabbits six-months-old vaccinated with 30 mg protein of Lot 7 vaccine without adjuvant 101

Figure 12 Agglutinin titer decline in one-year-old castrated males and ovario-hysterectomized females vaccinated with 30 mg protein of Lot 7 vaccine without adjuvant 103

Figure 13 Agglutinin titer decline in six-month-old castrated males and ovario-hysterectomized females vaccinated with 30 mg protein of Lot 7 vaccine without adjuvant 105

Figure 14 Linear regression of the overall agglutinin titer decline in male and female rabbits without regard of age, dosage, or vaccine lot 107

LIST OF TABLES

		Page
Table 1	Serum agglutinin titers in calves vaccinated with an acid-heat treated *Brucella abortus* Strain 19 culture vaccine	44
Table 2	Protocol for Experiment II. Box titration employing varying amounts of vaccine against varying challenge doses with four guinea pigs per letter group	48
Table 3	Protocol for Experiment III. Box titration employing varying amounts of vaccine against varying challenge doses with five guinea pigs per letter group	54
Table 4	*Brucella* agglutinin titers of guinea pigs vaccinated with 13-day acid-heat extracted whole culture vaccines 19 days post-vaccination (two days pre-challenge)	56
Table 5	Protocol for Experiment IV. Box titration employing varying amounts of vaccine against varying challenge doses with four guinea pigs per letter group	66
Table 6	*Brucella* agglutinin titers of guinea pigs vaccinated with 13-day acid-heat extracted whole culture and cell-free culture vaccines 18 days post-vaccination (three days pre-challenge)	67
Table 7	Vaccine and post-challenge *Brucella* agglutinin titers in non-vaccinated and vaccinated calves receiving 13-day whole culture and cell-free culture acid-heat extracted preparations	73
Table 8	Serum protein values of normal rabbits according to breed and sex	82

		Page
Table 9	Serum protein migration of normal rabbits expressed as a C/AF ratio	84
Table 10	Serum protein values of normal and abnormal rabbits	85
Table 11	Serum protein values of six to eight month old rabbits infected with *Eimeria stiedae*	87
Table 12	The effect of acid-heat extracted 13-day whole culture vaccines on the serum gamma globulin level in male and female rabbits . . .	91
Table 13	Analysis of variance of *Brucella* agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in rabbits one-year-old vaccinated with 20 mg protein of Lot 3 vaccine	96
Table 14	Analysis of variance of *Brucella* agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in rabbits six-months-old vaccinated with 20 mg protein of Lot 4 vaccine	98
Table 15	Analysis of variance of *Brucella* agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in rabbits one-year-old vaccinated with 30 mg protein of Lot 7 vaccine	100
Table 16	Analysis of variance of *Brucella* agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in rabbits six-months-old vaccinated with 30 mg protein of Lot 7 vaccine	102

Page

Table 17 Analysis of variance of Brucella agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in hormone treated castrated and ovario-hysterectomized rabbits one-year-old vaccinated with 30 mg protein of Lot 7 vaccine 104

Table 18 Analysis of variance of Brucella agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in hormone treated castrated and ovario-hysterectomized rabbits one-year-old vaccinated with 30 mg protein of Lot 7 vaccine 106

I. INTRODUCTION

The diagnosis of many infectious diseases is dependent upon the detection of antibodies circulating in the blood stream. In certain of these diseases the use of immunogenic agents produces confusing serological reactions. When the procedure for controlling such a disease utilizes vaccination, serological reactions due to the immunizing agent alone must not be considered as evidence of the presence of the disease. Brucellosis is an example of such a disease.

The two basic measures utilized in the United States in the attempts to eradicate bovine brucellosis are vaccination and the elimination of infected animals. The detection of infection relies upon the presence of specific agglutinins in the blood and milk (Blake and Manthei, 1952; Cameron, 1957; Soule, 1950) as a means of detecting infected animals. Unfortunately, agglutinins are not only produced by the naturally-occurring disease, but also by the use of vaccines (Manthei, 1953; Siero, 1954; Spink, 1956). It is recommended, therefore, that vaccines be administered during calfhood (Buck and Cotton, 1938) since the agglutinin titers subside by the time the animals reach adulthood in the vast majority of cattle (Birch *et al.*, 1945; Harring and Traum, 1943). Following this procedure it is theoretically possible to develop serologically negative herds and to assume that significant agglutinins developing after the animal reaches adulthood are a result of natural infection.

The fact that a titer due to the vaccine cannot be differentiated from one caused by the natural infection eliminates the use of revaccination in adult cattle to increase the protection against the disease. It is apparent that such a procedure would make it impossible to classify a titer occurring in an animal as one due to the naturally-occurring infection or to the vaccine.

Various methods have been devised (Barner *et al.*, 1952; Dick *et al.*, 1947; Kerr *et al.*, 1958; King *et al.*, 1953) in order to distinguish between vaccinated reactors and infected animals. These methods are not always reliable and are not feasible for use as routine diagnostic tools. A non-specific agglutinin has been described in normal sera (Hess and Roepke, 1951; Hess, 1953) at serum concentrations that tend to classify the animal as a suspicious or reactor animal.

The various vaccines that have been developed for the protection against brucellosis have one or more undesirable qualities, i.e., they may cause a lasting serum agglutinin response or provide inadequate protection of indeterminate duration. Up to the time that the present work was instituted, all effective brucellosis vaccines had been prepared from living or living-attenuated brucellae. Recently a killed cell vaccine has been developed in England (McEwen and Samuel, 1955).

II. REVIEW OF LITERATURE

Characteristics of Brucellae

The brucellae belong to the Order Eubacteriales and Family Brucellaceae, (Breed, Murray and Hitchens, 1957). This genus includes Brucella abortus, Brucella melitensis, Brucella ovis, Brucella suis and Brucella neotomae. These organisms are gram negative non-motile rods, which are parasitic and invade all animal tissues. They are pathogenic for various species of domestic animals and man (Spink, 1956). They may be grown in various enriched media between pH 6.1 and 7.4. Huddleson (1957) found that optimal growth of the carbon dioxide dependent strains occurs at pH 6.1 in the presence of suitable nutrients. He further demonstrated that the organisms may be grown in the presence of host blood alone when the pH is lowered sufficiently to destroy inhibiting substances present. Increased growth occurs in broth medium in the presence of 0.1 per cent agar. The function of the agar is of a physical nature only (Zobell and Meyer, 1932). Osmotic pressure of two to six atmospheres promotes the best growth (Braun, 1950) while pressures of 20 to 30 atmospheres are bacteriostatic (Zobell and Meyer, 1932).

Yields of 50 to 80 billion viable Br. suis cells per ml of medium are obtained, when growth takes place in aerated, enriched tryptose broth at 27.5°C (Braun, 1946). This is a very large number

and volume of cells as compared to Escherichia coli and Micrococcus aureus which yield one to two billion cells under similar conditions. Shake cultures have also been shown to be advantageous in increasing yields in broth cultures (Braun, 1950). Variation takes place in shaken liquid culture to a lesser extent than in static liquid culture over periods of growth of four to five days (Braun, 1946). A complete discussion of the nutrition of the brucellae may be found in the review by Gerhardt (1958) where composition of media, basic requirements, selective factors in population changes and practical applications are discussed.

Differentiation of the Brucella species depends mainly on dye sensitivity and hydrogen sulfide production. Final identification should be verified by the use of specific immune sera.

Variation and dissociation of the brucellae occur very readily in all species. Such characteristics as colony type, antigenicity, virulence, growth rates, viability, resistance to antibiotics, and dye tolerance are involved. Each one of these characteristics may vary independently, but there is a high degree of correlation of changes in certain characteristics. As an example, changes of colony type are associated with changes in antigenicity and virulence (Braun, 1950).

Dissociation is much more pronounced in liquid than on solid media. The smooth form may be maintained in liquid media by the addition of serum from a susceptible host (Braun, 1946).

Brucellosis in Cattle and Other Domestic Animals

The earliest reliable evidence of bovine brucellosis in the United States appeared in "The Cultivator" in 1843 (Evans, 1947). Heavy losses from abortion in cattle were reported in New York, Pennsylvania, Delaware and Virginia during this period.

Ten years after Bruce (1887) had isolated *Br. melitensis* as the causitive agent of Malta fever in humans, Bang (1897) a Danish veterinarian, successfully isolated the microorganism responsible for epidemic abortion in cattle.

The first reported isolation from cattle in the United States was made at the Illinois Experiment Station by MacNeal and Kerr (1910).

Brucella infection in the bovine tends to localize in the pregnant uterus, udder and lymph glands. In a gravid uterus, the placenta is attacked and the resulting inflammatory changes, if severe, cause the fetus to be aborted. When the inflammatory changes are mild the fetus may be carried to full term. This accounts for the fact that *Br. abortus* may be found in the placenta of blood test positive animals following normal parturition (Cotton, 1913). The organism usually disappears from the uterus within a period of 60 days following parturition (Cotton, 1913; Giltner and Bandeen, 1920). Udder infections, on the other hand, may persist for periods of up to five years (Schroeder and Cotton, 1911; Gwatkin, 1932).

The infection may enter through the vagina, mouth, skin or eyes (Bang, 1897; Birch and Gilman, 1925; Cotton and Buck, 1933; and Schroeder, 1922). The disease is spread by contact with aborted material, licking infected cows, and eating feed contaminated directly by discharges from infected cows, or by persons carrying infectious material on their shoes. Dust, flies, other animals and water may also serve as carriers of the disease.

The infected bull has been implicated in the transmission of brucellosis. Bendixen (1944) and Seit (1944) of Denmark have reported that infection occurred in previously clean herds when semen from infected bulls was used to artificially inseminate the cows. However, in a most comprehensive study King (1940) was unable to demonstrate this.

Swine and horses are susceptible to infection with Br. abortus, and can serve as sources of infection for cattle. Goats, mainly susceptible to Br. melitensis which is a close relation of Br. abortus, may also infect cattle.

Brucellosis of swine, commonly caused by Br. suis, was first diagnosed in the United States by Traum (1914). Many reports show that the disease is widespread and often causes serious losses from abortion. Br. suis differs from the Br. abortus in being more pathogenic for man and in not requiring an increased carbon dioxide tension

in the surrounding atmosphere for growth on artificial media. _Br. suis_ has been isolated from cows' milk (Hasseltine, 1930). Borts _et al._ (1943) have traced an epidemic of undulant fever in man to this organism in a milk supply.

Recently two new species of _Brucella_ have been isolated: one from sheep by Buddle (1953) in New Zealand, called _Brucella ovis_ and the other from rats isolated by Stoenner and Lackman (1957) called _Brucella neotomae_.

The economic importance of brucellosis may be estimated from the data collected from the University of Connecticut dairy herd during the period from 1914 to 1924. White _et al._ (1925) estimated a milk loss of $44.01 per year for each infected cow. In terms of milk production White _et al._ (1929) found the annual milk production per cow increased an average of 1,505 lbs. in a herd after the infection was eliminated. Minett and Martin (1936) found milk production in one infected herd was reduced 20.7 per cent, even though no abortions occurred.

Diagnosis

Little progress was made in the control of brucellosis until practical methods of diagnosis were developed, and it was recognized that all abortions are not due to brucellosis and that all infected cows do not abort.

The blood serum agglutination test has proved to be the most

accurate in the diagnosis of brucellosis in both animal and man. Wright and Semple (1897) first employed this method in the diagnosis of the disease in man, while Grinsted (1909) first used the technique for the diagnosis of the disease in cattle.

The approved procedure at present involves the use of standard methods plus a standard antigen of phenol-killed cells supplied by the United States Agricultural Research Service. This approach makes it possible for uniform results to be obtained in all laboratories using the approved methods and standard antigen.

Standard Serum Titer Interpretation

Serum Dilution				
1:25	1:50	1:100	1:200	Interpretation
- or i	-	-	-	Negative
+	i	-	-	Suspicious
+	+	-	-	Suspicious
+	+	+	i or +	Positive

Agglutination: - none, i incomplete, + positive.

Other common tests used in the diagnosis of brucellosis are: The rapid plate agglutination test, on blood serum (Huddleson and Carlson, 1926; Huddleson and Abell, 1928); agglutination test on milk whey (McFadyean and Stockman, 1916; Coolige, 1916); the milk ring test or Abortus Bang Ring test (Fleischhauer, 1937; Fleischhauer and

Herman, 1938: Roepke, 1950) and the vaginal mucus test (Kerr *et al.*, 1958).

Of the various methods mentioned above the blood serum agglutination test is now recognized as the most accurate procedure for the use in routine control programs. The ring test carried out at creameries on bulk milk is an important adjunct to the serum test. It is a method of screening the herds in an area and detecting those in which the infection may be present. The animals in these herds are then serum tested individually and the infected cattle identified. In a high percentage of herds the ring test has been positive when only one cow out of a group of five to fifteen animals was infected (Roepke, 1950).

The various other differential diagnostic tests employed in brucellosis are mentioned in the section, "Control of Brucellosis by the Use of Vaccines," since these tests are applicable in vaccinated animals only.

Control of Brucellosis in Cattle by the Use of Vaccines

Soon after Bang (1897) discovered the causative agent of infectious abortion of cattle, he attempted to prepare a vaccine against the disease. Bang (1906) reported that live virulent *Br. abortus* organisms when injected subcutaneously into cattle, prior to conception, prevented subsequent abortion. However, when toluol-killed cultures

were used little or no protection resulted. Vaccines prepared by killing cultures of Br. abortus with formaldehyde, mercurochrome, thionin and pyronin gave no protection (Gwatkin and Panisset, 1933). Similarly Br. abortus cells killed with chinosol, methylene blue, iodine and heat were found to be useless as immunizing agents (Zeller and Stockmeyer, 1936). More recently Schlingman and Manning (1952) reported favorable results using ultraviolet light irradiation of Br. abortus cells with subsequent precipitation by four per cent potassium alum. McEwen and Samuel (1955), employing a heat-killed rough strain 45/20 mixed with adjuvant, obtained a successful immunizing agent that was free of agglutinogens. McDiarmid and Sutherland (1957) have further substantiated these findings.

The earlier work of Bang (1906) followed by that of Stockman (1914) and McFadyean and Stockman (1916) indicated that viable cultures of Br. abortus were the most effective immunizing agents against brucellosis in cattle.

While vaccines containing living organisms have been shown to be the most effective, they can also be extremely dangerous. It is, therefore, mandatory that such a product conform to a rigid set of standards before it can be applied safely on a wide scale in the field.

The possibility of the immunogenic agent itself producing the disease in the animal that could be transmitted to man must be elimi-

nated. If the product is proven to be avirulent, it must then be demonstrated that it is capable of inducing effective immunity and will not revert to a more virulent form once it has been injected into the animal body. Finally the production of agglutinins as a result of the use of the vaccine must be evaluated so as to prevent any confusion in the interpretation of serological results. Such confusion would hamper eradication efforts by interfering with the basic diagnostic tool upon which the present control program relies.

The earlier-mentioned investigations with viable Brucella vaccines appeared so promising that the United States Bureau of Animal Industry licensed biological producers to make and distribute viable vaccines in 1919. However, these vaccines soon proved to be a dangerous source of the disease, producing a shedder state in the udder of many of the cattle vaccinated with this material (Hart and Traum, 1925). As a result of this finding, and the discovery that Br. abortus was pathogenic for man, the Bureau of Animal Industry since 1932 has restricted the production of vaccine, using only one specific strain of organisms known as Strain 19 (Traum, 1950).

Buck (1930) isolated an organism now known as Br. abortus Strain 19 from the milk of an infected cow. This highly virulent organism was attenuated by inadvertently leaving it on an agar slant at room temperature for a year. The virulence of this strain remained attenu-

ated even after repeated animal passage.

The Bureau of Animal Industry took an active interest in this strain and developed it into Strain 19 vaccine, which is currently used for the immunization of cattle in this country. In over twenty years of widespread use a persistent bovine infection has never been established using this vaccine. Four human cases have been observed in veterinarians who accidently inoculated themselves with this organism (Spink, 1957).

Strain 19 does dissociate and therefore rigid standards have been set by the United States Department of Agriculture for its production. Only 15 per cent dissociated organisms are allowed in a lot of vaccine. Each dose must contain ten billion viable organisms when packaged and five billion viable organisms at the expiration date (Traum, 1950).

Vaccination of cattle with Strain 19 produces sufficient immunity in 50 per cent of the animals to prevent infection from the usual natural exposure. A small group of five to ten per cent fail to develop serviceable immunity. Also, there is an intermediate group that develops a partial immunity, varying in degree between the two previously mentioned groups. The abortion rate in this intermediate group is low and the disease is minimal in intensity and duration (Birch and Gilman, 1945). Approximately five per cent of calfhood vaccinated animals may retain a suspicious or positive titer due to the vaccine.

Since vaccination can interfere with the use of the most important current diagnostic techniques, several methods have been devised to distinguish between titers indicating reactors due to vaccination and those due to natural infection. One of these methods is based on the anamnestic reaction. Barner _et al._ (1952) and Dick (1947) reported that vaccinated animals will respond promptly with a rise in agglutinins following an injection with strain 19. In contrast, a feeble response is produced in naturally infected animals. More recent studies by King _et al._ (1953) indicate this method is of doubtful value.

Traum and Maderious (1947) reported that agglutinins could not be demonstrated in the milk three months after the animals had been vaccinated, and Blake and Manthei (1952) reported that 98.9 per cent of cattle vaccinated as calves failed to show agglutinins in the milk with the whey agglutination test.

However, Roepke, _et al._ (1950) reported that 53 animals that were negative or at most one plus positive to the whey test, were positive to the blood serum agglutination test. _Br. abortus_ was isolated from 18 per cent of this group. Kerr _et al._ (1958) found two cows in a survey positive to the blood serum test that were on occasion negative to the whey test upon repeated sampling. On some occasions they were negative to the whey test yet showed positive _Brucella_ isolations. This would indicate that multiple biological examinations are neces-

sary to establish accurately the presence or absence of Brucella organisms in the udder. Relative to this, Cameron (1957) suggested that animals showing a titer of three plus or four plus to the whey test should be considered infected unless they were vaccinated within the past three months. Since whey two plus titers usually occur only in infected herds and these are subject to retest, such titers may be regarded as suspicious on original tests and positive if the titer persists on the retest.

A further method of differentiation is the vaginal mucus agglutination test. Data so far have demonstrated that tests on vaginal mucus are not influenced by the use of strain 19 vaccine injected subcutaneously (Kerr et al., 1958).

Presently available methods for differentiating titers due to the injection of a vaccine from those due to a natural infection are not only difficult and time consuming but are not sufficiently reliable to justify their routine use as part of the brucellosis eradication program.

Huddleson (1946) in his attempts to overcome the disadvantages of the strain 19 vaccine particularly that of the production of agglutinins in the vaccinates developed a mucoid vaccine, that was nonagglutinogenic.

Huddleson (1946, 1947, 1948) reported the isolation of a mucoid-

growth phase of Br. suis which had low virulence, good antigenicity, and did not provoke persistent titers or agglutinins in guinea pigs or cattle to the same extent as strain 19. Subsequent field studies and experimental investigations indicate that M vaccine is inferior to strain 19 in the protection produced against bovine brucellosis (Bryan et al., 1953). Results in cattle with the non-agglutinogenic Rosenbush vaccine (Rosenbush, 1947) used in Argentina have been similar (Siero, 1954). At the present time there is no evidence that M vaccine will revert to a more virulent form. However, other mucoid varients of Br. abortus and Br. suis have done so both "in vitro" and "in vivo" (Traum, 1950).

A more recent attempt to obtain a nonagglutinogenic vaccine with adequate protective qualities has been made by McEwen and Samuel (1955). It is a killed nonagglutinogenic type prepared from a rough strain 45/20 of Br. abortus. Preliminary results indicate that it is the first successful killed Brucella vaccine. The previously mentioned work of McDiarmid and Sutherland (1957) has substantiated their findings.

The various antigens obtained from brucellae that have also been used as immunogenic agents are included in the section, "Antigens of the Genus Brucella."

Antigens of the Genus Brucella

Investigations of the antigenic structure of the genus *Brucella* have been numerous and varied in their results. Wilson and Miles (1932) explained the serological differences between *Br. abortus*, *Br. melitensis* and *Br. suis* by the quantitative distribution of the two protein antigens A and M. The A antigen occurs in greater quantity than the M antigen in *Br. abortus* and *Br. suis* while the M antigen exceeds the A antigen in amount in *Br. melitensis*. Olitzki and Gurevitch (1933) explained the antigenic differences of these three strains similarly, but added a non-specific common antigen G. Renoux and Mahaffry (1955) described the antigenic composition of the brucellae in more detail. According to their scheme the smooth strains of the above three species contain the antigens A, M, Z and r in different quantitative distributions, while the ovine strain (Buddle, 1953) contains only Z and r. The rough strains are described as containing only r with or without Z. Olitzki and Sulitzeanu (1957, 1958) using the agar diffusion technique (Ouchterlony, 1948) found at least six antigens in sonic extracts of *Br. suis*. Sulitzeanu (1958), using this technique, found that sonic extracts of *Br. suis* contained seven antigens and that none of these antigens could be correlated with the presence or absence of agglutinins. Agglutinogens, however, were demonstrated in the insoluble cell debris.

In the search for those portions of the bacterial cells that are responsible for the immunological characteristics of the organisms, chemical fractionation techniques have been used extensively. Many attempts have been made to prepare immunologically active, soluble fractions of the brucellae. The most thorough studies reported in the literature are those of Miles and Pirie (1939 a, b, c, d) on _Br. melitensis_ and of Paterson, _et al._ (1947) on _Br. abortus._ Pennell (1950) has reviewed the many other chemical studies of the brucellae and these will be mentioned only as they relate to the work in the present study.

Miles and Pirie (1939 a, b, c, d) were able to prepare a fraction of _Br. melitensis_ as a highly complex material which they called "native antigen." This material was extracted from the cells by suspending the organisms in with two per cent phenol at 0°C for several days or weeks. The native antigen was separated into two main components called AP (amino polyhydroxy antigen) and a larger component called PLAPS (Phospholipid and Protein like-substance). The authors considered the AP fraction analogous to the SSS (specific soluble substance) isolated from the typhoid bacillus and to the "complete antigens" isolated by Boivin _et al._ (1933) from a number of gram negative organisms. Both antigens showed agglutinogenic activity in rabbits and guinea pigs. They were both capable of protecting mice from lethal toxic doses of these antigens (Miles and Pirie, 1939 d).

Paterson, *et al.* (1947) described a similar, but not identical, preparation from *Br. abortus*. Growth and extraction procedures were similar to those used by Miles and Pirie (1939 a). In its purified form the antigen was soluble over the entire pH range, but the material became insoluble after freezing. The antigen possessed significant immunizing properties in guinea pigs. Resistance was conferred with 0.3 mg of the material. Agglutinins were produced by as little as 0.3 gamma inoculated by various routes. However, the immunity produced by 0.3 mg to 0.6 mg of the material was no better than that produced by the administration of one dose of low virulence living cultures e.g., strain 19.

Huddleson (1943 a) prepared an antigen from the disintegration of living cells using a Booth-Green wet crushing mill. The crushed cells were diluted with distilled water. They were then shaken and centrifuged. Following this the top opalescent layer was removed. Examination of this material in the Tiselius electrophoresis apparatus revealed three minor and one major component. This preparation was an effective immunogenic agent, protecting guinea pigs from subsequent infection without development of high agglutinin titers. Its immunologic properties were destroyed by 1:10,000 concentration of merthiolate, or when held at a temperature of 55°C for 30 minutes.

Vanghelovici *et al.* (1938) applied the trichloroacetic acid extrac-

tion procedure of Boivin *et al.* (1933) to *Br. abortus*, *Br. melitensis* and *Br. suis*. They produced extracts of the three species by suspension of the washed organisms in 0.25 N trichloroacetic acid. These substances were toxic and produced marked serological activity with both homologous and heterologous antiserum.

Soluble Extracellular Immunogenic Agents

Much of the information on protective substances found in culture filtrates has been gained as a result of the work done on anthrax (Gladstone, 1946, 1948; Wright *et al.*, 1954; Puziss and Wright, 1954; Belton and Strang, 1954; Thorne and Belton, 1957; Boor and Tresselt, 1955).

Maitland and Guerault (1958) demonstrated that a soluble mouse-protective antigen, a histamine sensitizing factor, and an agglutinogen may be obtained by washing *Hemophillus pertussis* cells with saline. They found that when the organisms were grown on Bordet-Gengou medium the histamine sensitizing factor and mouse-protective antigen diffused into the medium.

Hoag and Bell (1955) demonstrated that a soluble, acid-heat, extracted vaccine gave protection against *Leptospira pomona* in cattle. Further investigations of this agent have indicated that methods of growth, preparation and time of growth are all involved in the produc-

tion of a suitable agent of this type (Hoag and Allen, 1959).

Recently the presence of a soluble, protective antigen has been demonstrated in the brains of mice infected intra-cerebrally with the flury strain of rabies virus. Van den Ende et al. (1957) demonstrated that the maximum concentration of both the antigen and the virus occur at the same time. It can be purified by precipitation and is stable at 56° C. Heating, however, markedly reduces stimulation of neutralizing antibody formation.

While the previously-mentioned work does not include all reports relative to soluble extracellular antigens; it serves to indicate that bacteria, both gram negative and gram positive, spirochetes, and viruses are capable of producing antigens that are distinct and separate from the microorganism itself.

III. OBJECTIVE OF STUDY

Brucella vaccines composed of metabolic by-products have not been investigated. Recent studies with a soluble *Leptospira pomona* vaccine indicated the feasibility of such vaccines. The primary purpose of this study was to produce a soluble immunogenic agent using simple chemical procedures which would be free, or essentially free, of agglutinogens.

Data are presented which show that an immunity may be produced in guinea pigs against *Brucella abortus* employing a solubilized vaccine obtained from an acid-heat extracted whole culture. The data further indicate that an equally strong immunity may be produced with a soluble vaccine prepared from an acid-heat extracted cell-free culture. This cell-free by-products vaccine produces little or no agglutinin response. The immunological response of guinea pigs, rabbits and calves to the two types of soluble vaccines are presented. The possible roles of sex and age in the response of rabbits to the soluble whole culture vaccine are discussed.

IV. METHODS AND MATERIALS

In order to avoid confusion and the need for cross reference, only general methods and materials are discussed here. Any modifications of the general methods are given in detail in each experiment where they apply.

A. Brucella Cultures:

Brucella abortus Strain 19 was obtained from commercial strain 19 live vaccine (Lederle Laboratories division of American Cyanamid Company, Pearl River, New York) by culturing it on agar plates. *Brucella abortus* Strain 2308 was kindly supplied by Dr. Archie Frank of the Agricultural Research Service, Beltsville, Maryland.

B. Media:

The basal media for all *Brucella* recoveries was Brucella Broth (Albimi Laboratories, Incorporated, Brooklyn 2, New York). For primary isolations and viable counts, 1.5 per cent agar (Difco) and a 1:50, 000 concentration of basic fuchsin (National Aniline Division, Allied Chemical and Dye Corporation, New York, New York) was added to the basal medium to prepare agar plates. Semi-solid agar, prepared by adding 0.1 per cent agar (Difco) to the basal medium, was used for primary isolations from blood and organ material. Huddleson's (1957) host blood medium was also used for

primary blood recovery from cattle. All soluble vaccine cultures were grown in Stuart's medium (Stuart, 1946) containing 10 per cent rabbit serum.

Serum for media was prepared in the following manner. Pooled rabbit blood was collected in one-gallon jars and was allowed to clot for 12 to 24 hours at 4°C. The free serum and cells were decanted and centrifuged at 850 x G for 10 minutes to sediment the cells. The supernatant serum containing some hemolysis was decanted and passed under pressure through a Seitz clarifying filter. The serum was sterilized by passing it through a sterile Selas candle filter of 0.03 porosity. Sterility was checked by incubation of three 1.0 ml aliquots of serum in nine ml of thioglycollate broth (Difco) for 48 hours. Stuart's medium, following the aseptic addition of sterile rabbit serum, was incubated 48 hours as a sterility check on the completed medium.

The compositions of all media used are given in the appendix under media.

C. Brucella recoveries:

Animals were stunned by a blow on the head and sacrificed by exsanguination. Organs to be examined bacteriologically were removed aseptically and placed in sterile containers. The organ surface was seared by a hot spatula and a flamed bacteriologic loop

was inserted through the seared area. One loopful of material so obtained was used to inoculate a tube of semi-solid medium and an agar plate. A portion of the organ was then removed with alcohol-flamed instruments and used to streak an agar plate.

In order to reduce the oxygen tension of plate cultures for optimum growth, a lighted candle was placed on top of the Petri-plates in a steel canister and the lid was replaced and sealed with adhesive tape. The tubes containing inoculated semi-solid media were placed in dessicators, after loosening the caps, and the atmosphere was flushed with 10 per cent carbon dioxide. All inoculated plates and tubes were incubated for five days at 37.5°C. Brucella isolates recovered from the experimental animals were tested serologically against cross absorbed human and rabbit sera specific for strain 2308 (Huddleson, 1943b).

D. Agglutination Test:

The Brucella agglutinin titers of all experimental animals were determined by the standard tube agglutination test of Fitch *et al.* (1930). All titers were expressed as the final dilution of the last tube showing agglutination. Antigen for *Brucella abortus* Strain 2308 was prepared after the method suggested by McCullough (1950), with the exception that the organisms were cultured on the basal agar medium. Smoothness of cultures was confirmed visually by the use

of reflected light (Henry, 1933; Huddleson, 1951) and by the saline antiagglutination test (Elberg, 1948). Each lot of antigen was tested with known positive and negative serum and compared to the standard tube agglutination test antigen (Br. abortus Strain 1119-3, supplied by the United States Department of Agriculture, Agricultural Research Service, Animal Disease Eradication Branch, Washington 25, D. C.).

E. Bacterial Counts:

Total viable counts were estimated by making ten-fold serial dilutions in the basal medium. Immediately following dilution, duplicate drop agar plates were prepared by dropping 0.05 ml amounts of each dilution on the center of agar plates and spreading the drop evenly over the surface by rapidly rotating the plate at an angle of 45 degrees. Total Brucella cell concentrations were estimated by turbidity measurements in a "Spectronic Twenty" (Bausch and Lomb) using the method of Demoss and Bard (1957).

F. Brucella Challenge Organisms:

Brucella challenge organisms were obtained from smooth, single, colony isolates from the second media passage of infected guinea pig spleens. A colony was inoculated onto three to four basal medium agar slants and the slants were incubated for 48 hours. Organisms were washed from the slant with saline and checked with the slide agglutination test, and microscopically with the gram stain. The

washings were pooled and made up to a concentration approximating the first tube of the McFarland nephelometer series. Serial ten-fold dilutions of this material were made in physiological saline and these served as challenge material.

G. Serum Preparation for Serology and Electrophoresis:

Rabbits and guinea pigs were bled by cardiac puncture. Eight ml of blood were removed from rabbits using a one and one-half inch 20 gauge needle and 10 ml syringe, while two ml of blood were taken from guinea pigs using a one and one-half inch 22 gauge needle and a five ml syringe. The syringes and needles were rinsed prior to bleeding with a 2.5 per cent sodium citrate solution, which was found to be the best method for the prevention of red cell hemolysis. Bovine blood was removed from the jugular vein using California bleeding needles inserted into standard cattle bleeding tubes. All blood was allowed to clot for a period of three to four hours at room temperature, after which time serum and any free cells were withdrawn by a capillary pipette and placed in conical 15 ml centrifuge tubes. The serum, and free cells were centrifuged for 15 minutes of 200 x G. The clear supernatant serum was collected in capillary pipettes and placed in five ml screw cap vials and stored at -5°C.

H. Paper Electrophoresis:

Hanging strip paper electrophoresis was employed using Durrum-

type cells. All separations were carried out using barbiturate buffers at pH 8.6. The varying ionic strengths, currents and separation times employed are given in detail in the experiments where they were used. Rabbit serum was separated using the standard method for human serum (Spinco Technical Bulletin 6027A, 1955). Vaccines and bovine sera were separated employing the method of Rooney (1957). Strips were stained using the aqueous bromphenol blue procedure and were then analyzed on an "Analytrol Model R" servo type integration scanner. The equipment, buffers, fixatives and stains were all purchased from The Spinco Division of Beckman Instruments Incorporated, Palo Alto, California.

I. Chemical Tests:

The protein and non-protein nitrogen content of cultures and vaccines were determined quantitatively using the Micro-Kjeldhal technique (Hawk, Oser and Summerson, 1947). The Molish test was used for the detection of carbohydrate in the vaccines (Hawk, Oser and Summerson, 1947).

J. Care and Handling of Animals:

Male guinea pigs approximately 200 grams in weight were obtained from Albino Farms, Red Bank, New Jersey. They were placed in groups of six to eight in sterilized, galvanized, metal rabbit cages housed in large animal isolation rooms. A therapeutic level of 25

mg per animal of oxytetracycline was given in the drinking water for 72 hours after arrival in an attempt to prevent the outbreak of intestinal and respiratory infections. Guinea pigs were fed a commercial, pelleted, rabbit feed supplemented with six ounces of corn oil and a tablespoon of meat meal per bowl of feed. Cabbage and lettuce were supplied daily as a source of vitamin C. At times when there was a shortage of greens, vitamin C powder at a level of 25 mg per animal was dissolved in 10 ml of water and sprinkled directly on the feed. Water, feed and greens were given ad libitum.

Rabbits were raised outdoors on wire until use. They were then placed individually or by the same sex in pairs in rabbit cages. housed in large animal isolation rooms. The rabbits were fed a commercial, pelleted, rabbit feed. Cabbage and lettuce greens, unmedicated salt, and fresh water were also supplied ad libitum. All rabbits were checked for gastrointestinal parasites prior to experimental use by Dr. D. F. Watson of this department. Serum protein patterns were determined using paper electrophoresis at weekly intervals for a period of two to three weeks prior to the start of any experimental work. Each rabbit was sacrificed either at the end of the experimental work or upon the determination of an abnormal serum protein pattern. Histological sections were made on all pathological material found at necropsy.

Male calves were obtained at birth from the Department of Dairy Science, Virginia Polytechnic Institute and were raised in wooden pens for the first nine weeks of life. During this time they were fed increasing amounts of milk fortified with a mineral and vitamin supplement. Pelleted calf feed was given as soon as the calves would accept it. After nine weeks the calves were pastured in holding lots and fed alfalfa hay, water, and salt on a free choice basis. A mixed blackleg, gas gangrene bacterin (Lederle Division of American Cyanamid Company, Pearl River, New York) was administered at 10 to 12 weeks of age. Therapeutic levels of 20 gm of phenothiazine (drench grade) in two per cent copper sulphate per 100 pounds of body weight were given for the control of gastrointestinal parasites prior to the experiments. Serological examination for Brucella agglutinins and serum protein patterns were determined at weekly intervals for a period of three weeks prior to vaccination.

All challenge work was conducted in specially prepared large animal isolation rooms under strict isolation conditions. Prior to Brucella challenge, the isolation rooms were sealed on the inside and iodine treated gauze pads filled with cotton were placed over the exhaust outlet. After infection the following additional precautions were employed to prevent the dissemination of the live

organisms. Separate coveralls and boots were placed in the entry room for use in the isolation room. Rubber gloves and disposable gauze masks filled with non-adsorbent cotton were worn at all times when in the isolation rooms. Gloves and boots were washed and the masks were discarded in a cresol solution contained in foot trays in the entry room upon leaving the isolation room. Boots, gloves, and coveralls were removed in the entry room prior to reentering the main hall. Fecal material was removed in double sealed containers and sterilized before being taken to the incinerator. All visitors and persons not directly concerned with the experiments were prohibited from entering any isolation room. This was done to reduce the chance of spreading Brucella infections and to prevent the introduction of any extraneous infectious diseases.

V. EXPERIMENTAL RESULTS

A. Selection of an Effective Method for the Preparation of a Soluble Brucella Vaccine.

Experiment I. A Preliminary Study of an Acid-Heat Treated Brucella Abortus Strain 19 Vaccine in Calves.

Procedures: *Brucella abortus* Strain 19 was grown in trypticase soy broth (BBL) containing 0.1 per cent agar (Difco). After 48 hours incubation, the culture was heated to boiling and N/10 acetic acid equal to three per cent of the culture volume was added (Hoag and Bell, 1955). Boiling was continued for 10 minutes and then the material was cooled to 4°C and held at this temperature overnight. The resultant gelatinous precipitate was harvested by centrifugation at 2,500 X G for 30 minutes at 4°C. The gelatinous material was then reconstituted to one-fifth of the original culture volume with N/10 sodium hydroxide. This material was designated the raw vaccine. The raw vaccine was mixed with an adjuvant consisting of 12 per cent (v/v) lanolin in mineral oil (Freund, 1948), in a ratio of three parts of adjuvant to two parts of raw vaccine.

This type of a water in oil emulsion was chosen for its ability to prolong the action of the antigen and for its enhancing affect due to local irritation, which presumably increases antibody response.

Five ml of the vaccine was injected into eight one-year-old heifer calves, previously determined to be free of Brucella agglutinins using standard tube agglutination test antigen. The vaccine was injected subcutaneously into the cervical area. Titers were determined for 20 weeks post-vaccination.

Results: The Brucella agglutinin response to the vaccine, which is shown in table 1, indicated that this material elicited titers in all of the animals. The response in heifers one and four was minimal and their highest level was a suspicious titer of 1:80. The other six animals showed titers that may be considered as positive by federal regulatory standards; i.e., 1:160 or higher using the dilution series employed. These were apparent at one or more test periods up to four to eight weeks following vaccination. Animal three still showed a positive titer level 20 weeks following vaccination. The geometric mean titers for the group indicated that the maximum titer level was produced between one and four weeks following vaccination and declined slowly to yield a geometric mean titer approximately one-half of the peak titer at 20 weeks post-vaccination.

Five of the eight animals were still in the suspicious range and one was in the positive range at the conclusion of the 20 week observation period. No challenge studies could be attempted as

TABLE 1

Serum agglutinin titers in calves vaccinated with an acid-heat treated *Brucella abortus* Strain 19 culture vaccine.

Calf number	Agglutinin titer(1)					
	Pre-vaccination -14 to -2 days	Post-vaccination weeks				
		1	4	6	8	20
1	-(2)	20	80	80	80	40
2	-	-	160	160	160	80
3	-	80	160	160	640	160
4	-	-	80	80	80	80
5	-	20	320	320	80	80
6	-	-	320	160	160	20
7	-	20	80	160	40	80
8	-	20	320	80	80	80
Geometric mean titer	-	15	160	136	120	68

(1) Titer expressed as reciprocal of the dilution.

(2) Negative at 1:20, the lowest test dilution employed.

these animals were scheduled for other experimental work.

The use of agar to increase the yield of organisms (Zobell and Meyer, 1932) proved to be undesirable in the vaccine. The agar caused the vaccine to be of a gelatinous rather than a liquid nature which made further purification difficult. This feature, as well as the production of agglutinins, excluded this method of preparation from further consideration in the search for methods of preparing a soluble agglutinogen-free immunogenic agent.

Experiment II. Preliminary Study of an Acid-Heat Extracted 48 Hour Growth Culture Vaccine in Guinea Pigs.

Stuart's medium was tested next to determine its suitability for a vaccine media. It contains no agar, and the rabbit serum serves to promote growth and maintain the brucellae in the smooth form (Braun, 1946; Huddleson *et al.*, 1945). It was also decided that a strain of *Brucella* highly pathogenic for cattle would be a more desireable organism from which to obtain soluble metabolic antigens than was the attenuated strain 19. *Brucella abortus* Strain 2308 was obtained for further work, since its virulence for cattle was known (Manthei, 1950).

Procedures: *Brucella abortus* Strain 2308 was grown in Stuart's medium for 48 hours at 37.5°C under a 10 per cent carbon dioxide atmosphere. The culture, containing 1.0×10^8 viable organisms per ml, was then heated to boiling at which time N/10 acetic acid was added in an amount equal to five per cent of the total culture volume. Boiling was continued until precipitation was visibly complete. The material was cooled to 4°C and the precipitate was obtained by filtration using Whatman number one filter paper. The precipitate was dissolved by the addition of N/10 sodium hydroxide and then brought to one-fifth of the original culture volume with

distilled water. Merthiolate* was added as a preservative in a final concentration of 1:10,000. This material constituted the raw vaccine to which was added an adjuvant of six per cent lanolin in mineral oil (v/v) in a similar ratio to Experiment I. Six per cent lanolin was adopted to decrease the visocity of the final product in order to make the vaccine easier to inject. The droplet size of the final emulsion was not adversely affected by the decrease in lanolin concentration.

Sixty-four male guinea pigs weighing approximately 250 grams each were randomly divided into 16 groups of four animals each for a box type titration as illustrated in table 2. Animals receiving vaccine were injected subcutaneously in the cervical region. Twelve days following vaccination the animals were challenged by subcutaneous injection of ten-fold serial dilutions of Brucella abortus strain 2308. One ml amounts of challenge corresponding to 3.0×10^1, 3.0×10^3, 3.0×10^5 and 3.0×10^7 viable organisms were used as shown in table 2. All guinea pigs were bled for Brucella agglutinin titers prior to vaccination, two days before challenge (10 days post-vaccination) and again 28 days

*Thimerosal, Eli Lilly & Co.

TABLE 2

Protocol for Experiment II. Box titration employing varying amounts of vaccine against varying challenge doses with four guinea pigs per letter group.

Number of viable organisms in challenge*	Amount of vaccine ml			
	None	0.25	0.5	1.0
3×10^7	A	E	I	M
3×10^5	B	F	J	N
3×10^3	C	G	K	O
3×10^1	D	H	L	P

* The undiluted challenge material contained 3×10^8 viable organisms per ml.

post-challenge at the conclusion of the experiment.

Animals were observed for 28 days after receiving the challenge organisms and any deaths or abnormalities were noted. The 28 day period was chosen since maximum pathology and Brucella recoveries occur in guinea pigs between four and five weeks following infection (Smith and Fabyan, 1912; Braude and Anderson, 1950). All animals were bled, sacrificed and the organs were cultured for Brucella recoveries at the conclusion of the experiment.

Results: Seven animals died during the experimental period and are not included in the results. Of these, three appeared to have died of a nutritional deficiency, three had caught a leg in the wire bottom of the cage and struggled to death and no cause could be attributed to the death of the last animal.

Protection results from Lot 2 vaccine are shown in figure 1. Titration of the challenge material in the non-vaccinated controls resulted in infection of 50 per cent of the animals (ID_{50}) by 1.64×10^4 viable Brucella organism based on the Reed-Muench method of determining 50 per cent infection (Reed and Muench, 1938). The animals receiving 0.25 ml of raw vaccine plus adjuvant demonstrated an ID_{50} of 6.86×10^4 viable organisms; while the animals receiving 0.50 ml and 1.0 ml of the product demonstrated

ID_{50}'s of 3.91×10^4 and 6.83×10^4 viable organisms respectively. The guinea pigs receiving the 0.25 ml dosage of vaccine showed 50 per cent infection at a challenge level of 0.5 log less than the non-vaccinated controls; while the animals receiving 0.5 and 1.0 ml of vaccine required 0.2 log and 0.5 log respectively more viable organisms to infect 50 per cent of the animals. Since a difference of two logs or greater is considered evidence of protection, no significant protection was offered by any of the dosage levels of this vaccine.

No agglutinin titer response was elicited in any guinea pigs by the various dosages of vaccine 10 days following vaccination; thus, indicating that no detectable agglutinogenic material was present. At the end of the experimental period there was a marked agglutinin titer suppression in the vaccinated animals as compared to the non-vaccinated controls (figure 1). This might indicate that there was some protective action produced by the vaccine based on the assumption that the agglutinin level is proportional to the amount of antigen and conversely to the number of organisms present.

The protection and titer results indicated that the vaccine might be effective provided that certain conditions of the experiment were modified. It had been shown in previous work with immunogenic

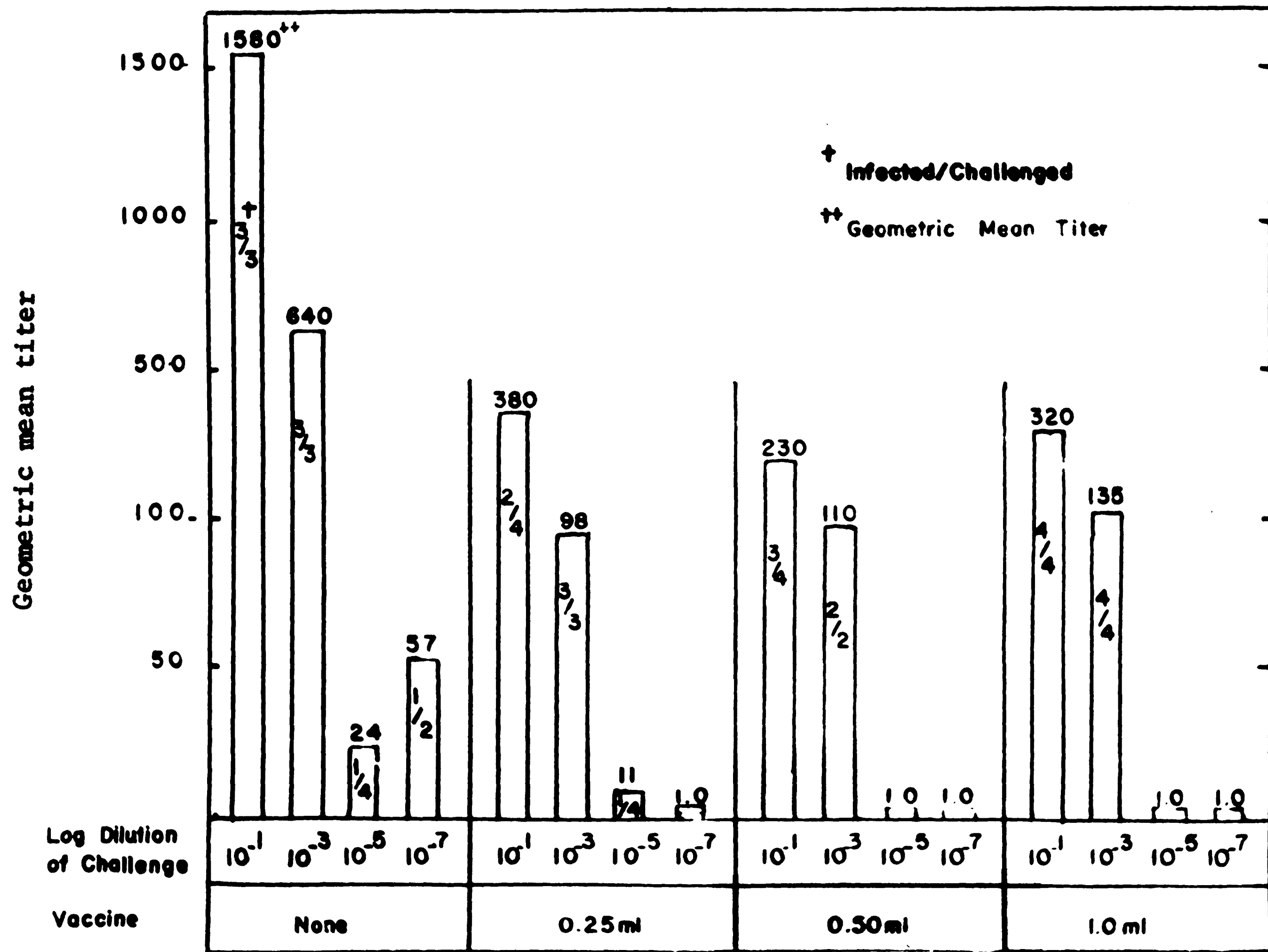

Figure 1. Geometric mean titers and Brucella abortus recoveries 28 days post-challenge from vaccinated and control guinea pigs. Experiment II. Results obtained from guinea pigs vaccinated with 48-hour whole culture acid-heat extracted vaccine with four guinea pigs per group at start.

agents consisting of metabolic products that the age of the culture played an important role in the liberation and amount of such products (Hoag, 1955). It was also possible that the period between vaccination and challenge was too short to have produced an effective immunity with such a product. Both possibilities were considered in the following experiment.

Experiment III. Preliminary Studies on an Acid-Heat Extracted 13-Day Growth Culture Vaccine.

Procedures: *Brucella abortus* Strain 2308 was grown in Stuart's media for 13 days, since the shorter incubation period apparently did not liberate sufficient metabolic products into the medium. The 13-day culture, containing 1.0×10^7 viable organisms per ml, was treated in an identical manner as Experiment II and was designated Lot 3 vaccine.

The only differences between procedures used in this experiment and Experiment II were that the dosage and challenge levels were changed slightly and that there were five guinea pigs per group at the start of the experiment (see table 3). The animals in this experiment were challenged 21 days post-vaccination rather than 12 days post-vaccination as in the previous experiment.

Results: Four animals died within the first 10 days following vaccination. Three animals had caught a leg in the wire bottom of the cage and apparently struggled to death and one death was due to an undetermined cause.

On the basis of Brucella recoveries 28 days-post challenge (shown in figure 2) it may be observed that an ID_{50} was accomplished in the control animals by 3.4×10^4 viable organisms. At all levels

TABLE 3

Protocol for Experiment III. Box titration employing varying amounts of vaccine against varying challenge doses with five guinea pigs per letter group.

Number of viable organisms in challenge*	Amount of vaccine ml			
	None	0.25	0.50	0.75
1.2×10^6	A	E	I	M
1.2×10^5	B	F	J	N
1.2×10^4	C	G	K	O
1.2×10^3	D	H	L	P

* The undiluted challenge contained 1.2×10^8 viable organisms per ml.

of vaccine dosage the ID_{50} was equal to or greater than 3.4×10^6 viable organisms. The protection produced in the vaccinates (difference between non-vaccinated controls and vaccinate ID_{50}'s) was two logs or more, which indicated that significant immunity was produced.

All sera from guinea pigs in the groups receiving the 0.25 ml dosage of immunogenic material and adjuvant had a tube agglutination titer of less than 1:20 when tested 19 days after vaccination (two days prior to challenge see table 4), with the exception of guinea pig number 11 which showed a titer of 1:40 at this time. In the group of animals receiving 0.5 ml of the immunogenic agent and adjuvant there were a greater frequency of agglutinin titers produced. Animals four, eight, fourteen and seventeen all showed titers. In the groups of guinea pigs receiving the largest dosage of immunogenic agent and adjuvant there were only four animals that did not show titers. Only animals one and fourteen receiving 0.75 ml vaccine demonstrated a marked titer response however.

The titer response in the vaccinated animals 28 days post-challenge indicated a marked titer suppression effect which further indicated protection. In the animals receiving the highest dosage of vaccine there was an indication of an anamnestic titer response. This is shown by the higher titers in this group as compared to the

TABLE 4

Brucella agglutinin titers[1] of guinea pigs vaccinated with 13-day acid-heat extracted whole culture vaccines 19 days post-vaccination (two days pre-challenge).

Guinea pig number/group	Amount of vaccine ml		
	0.25	0.50	0.75
1	-[2]	-	160
2	-	-	20
3	-	-	-
4	-	80	80
5	-	-	20
6	-	-	20
7	-	-	40
8	-	40	20
9	-	-	40
10	-	-	-
11	40	-	40
12	-	-	-
13	-	-	40
14	-	80	160
15	-	-	80
16	-	-	40
17	-	80	20
18	-	-	-
19	-	-	40
20	-	-	-
Geometric mean	1.5	7.5	26.0

(1) Titer expressed as reciprocal of dilution.

(2) Negative at 1:20, the lowest test dilution employed.

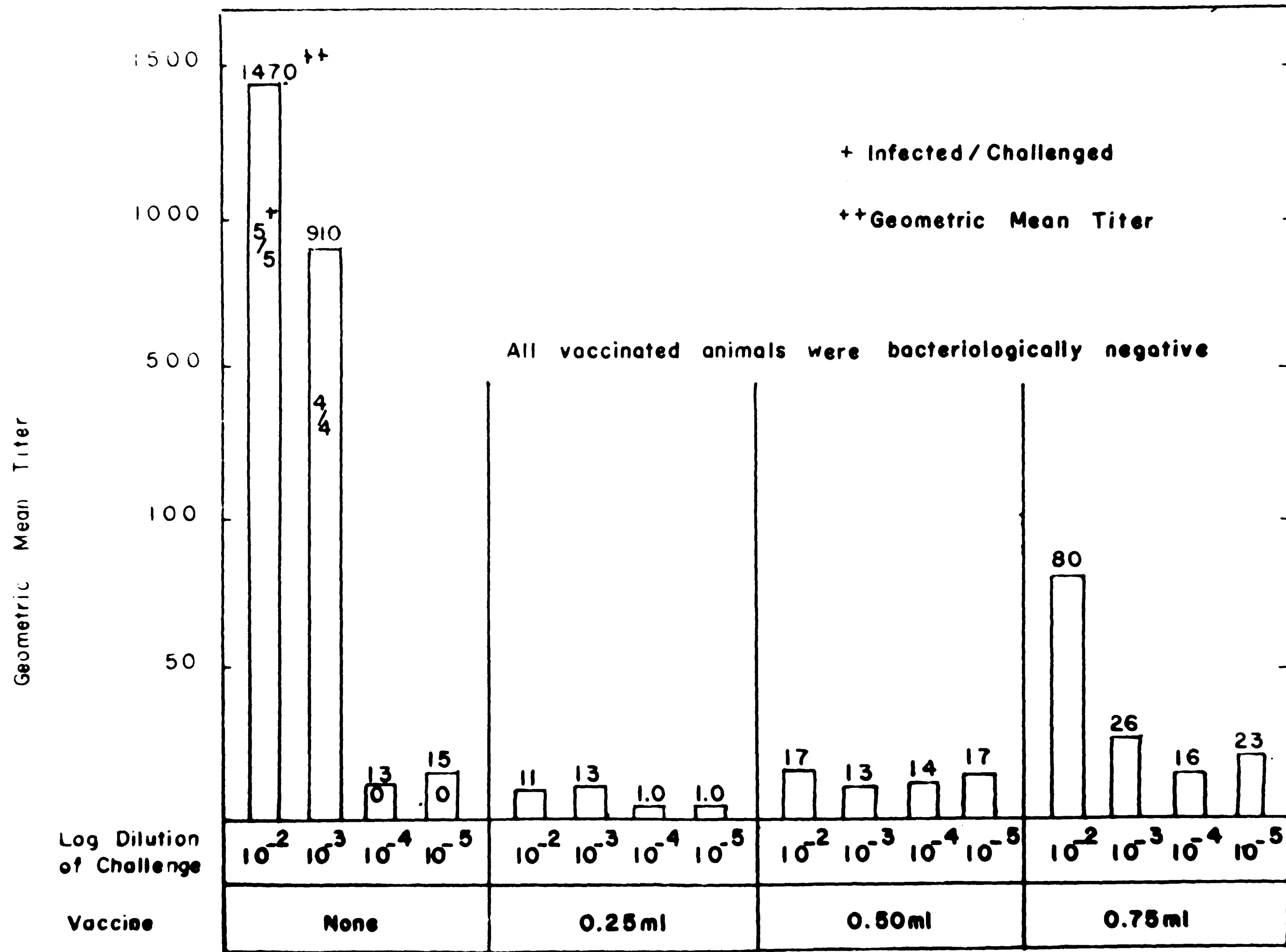

Figure 2. Geometric mean titers and Brucella abortus recoveries 28 days post-challenge from vaccinated and control guinea pigs. Experiment III. Results obtained from guinea pigs vaccinated with 13-day whole culture acid-heat extracted vaccine with five guinea pigs per group at start.

vaccinates receiving 0.25 ml and 0.5 ml of the immunogenic agent. The greatest agglutinin response was the group where the highest challenge dose and highest dosage of vaccine were employed.

The degree of immunity produced by the vaccine in this experiment leaves little doubt that an effective immunogenic agent for guinea pigs could be produced by this method of preparation. In addition, it was shown that immunogenically effective levels of the material did not evoke a significant vaccine agglutinin response. The larger dosages of the 13-day culture vaccine produced agglutinin titers as well as immunity. These were not evident, even using larger dosages, in the 48-hour culture vaccine. A similar number of viable organisms were present in each culture at the time of vaccine preparation, which indicated that the immunogenic agent and agglutinogenic material did not arise from the viable brucellae alone.

These findings indicated that metabolic by-products in the older culture probably contributed the major share of both agglutinogenic and immunogenic materials to the vaccine.

B. Characteristics and Purification of Acid-Heat Extracted Soluble Antigens and Their Responses in Guinea Pigs and Calves.

In Experiment III the 13-day whole culture vaccine produced agglutinins in guinea pigs when given at high dosage levels. Results obtained in Experiments II and III indicated that the amount of by-products in the culture was the determining immunogenic factor and that the number of viable organisms was unimportant. The purpose of the following experiment was to determine if some of the agglutinogens could be removed from the vaccine by removing the intact Brucella cells. If this procedure did not interfere with the effectiveness of the vaccine, it would lend further support to the basic premise that soluble metabolic by-products may act as immunogenic agents. It would also be one step further in the purification of the material. Growth and chemical studies were performed at intervals throughout the culture growth period to determine what changes were occurring.

Experiment IV. A Comparison of Soluble Acid-Heat Extracted Whole Culture and Cell-Free Culture Brucella abortus Vaccines.

Procedures: One batch of Stuart's media was divided into four 220 ml aliquots which were transferred aseptically into 250 ml flasks. An additional 1100 ml aliquot was transferred aseptically into a two liter flask. Three small flasks were inoculated with 0.2 ml of a 48 hour growth culture of *Br. abortus* Strain 2398, and the fourth served as an uninoculated control. The larger flask was inoculated with 1.0 ml of the same culture material. The inoculum was made up to a concentration approximating the third tube in the McFarland nephelometer series. The inoculum was checked in a manner similar to the challenge material. Flasks were removed from the incubator at zero, three, six, nine and thirteen days. Each flask was treated as follows: Total viable count, turbidity, total protein nitrogen and non-protein nitrogen were determined at each interval on aliquots of the material. The remainder of the culture was divided in half; one-half was treated similarly to the procedure in Experiments II and III, the remaining half was passed through a Seitz EK filter to remove the brucellae prior to acid-heat extraction. Protein nitrogen, non-protein nitrogen and paper electrophoretic analyses were performed on both

types of raw vaccines.

The buffer system used for paper electrophoresis was sodium-barbital-barbituric acid with an ionic strength of 0.053 and a pH of 8.6. The preparations were applied in 0.04 ml amounts to Whatman 3 MM filter paper strips and separations were carried out for five hours at a constant current of 21 ma.

Eighty 250 to 300 gm male guinea pigs were randomly divided into twenty groups of four animals each for a box type titration as illustrated in table 5. Vaccinated groups were injected subcutaneously in the cervical region. Twenty-one days following vaccination the animals were challenged by intraperitoneal injection of 5.44×10^5, 5.44×10^6, 5.44×10^7 or 5.44×10^8 viable organisms. The intraperitoneal route of challenge was chosen in order to subject the vaccines to a more severe test. All animals were bled for Brucella agglutinin titers prior to vaccination and at the end of the experiment. Post-vaccination agglutinin titers were determined in a separate group of animals and these results are shown in table 6.

Results: Growth studies on the Brucella cultures used to prepare the vaccine are shown in figure 3. The results indicated that the maximum viable count was followed closely by maximum turbidity. If one assumed that the maximal concentration of metabolic by-products are liberated during the logarithmic death phase, then betwen 10 and 13 days

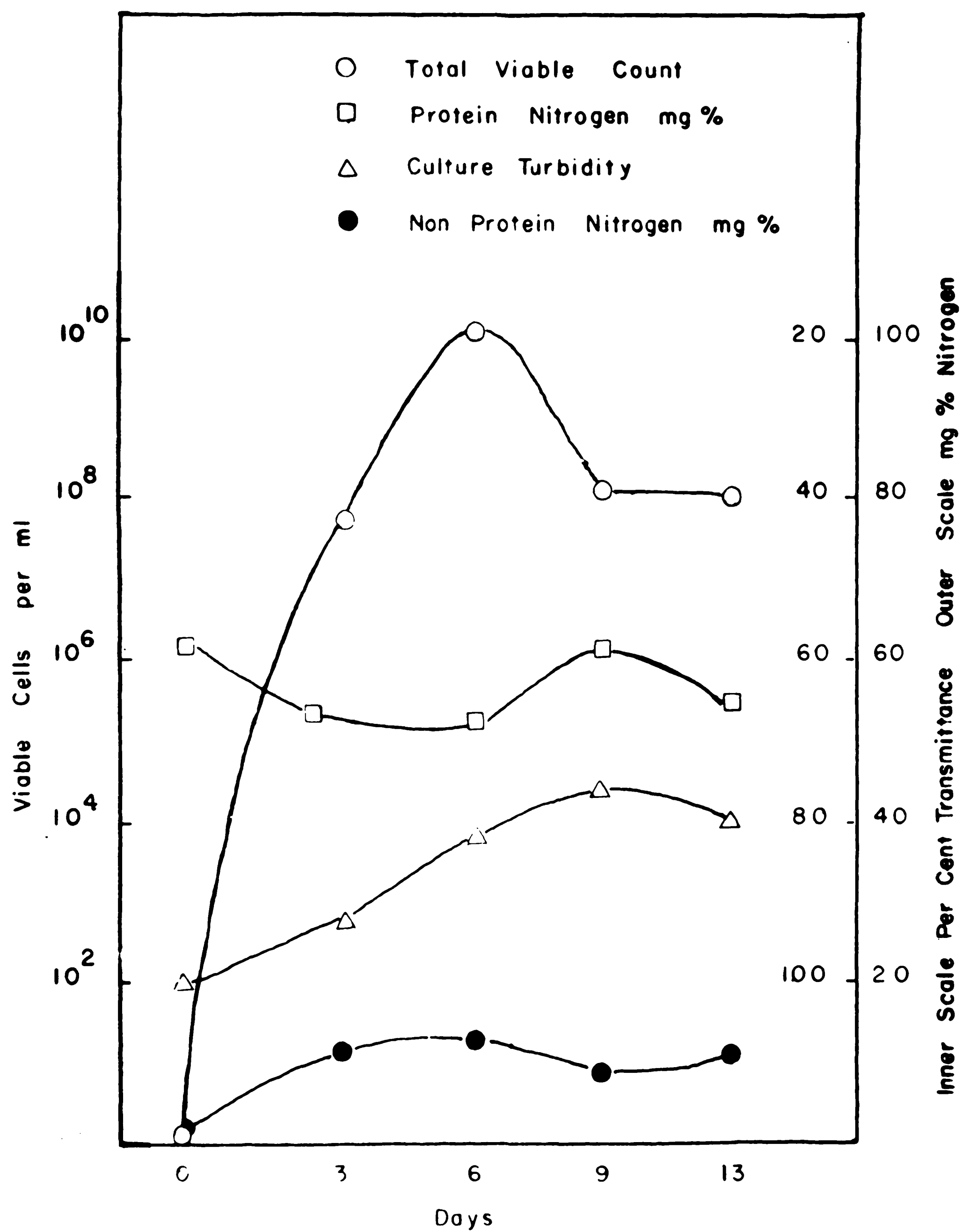

Figure 3. Growth response studies of Brucella abortus Strain 2308 grown in Stuart's medium containing 10 per cent rabbit serum.

would appear to be the optimum period of incubation before harvesting the vaccine culture. Total protein nitrogen and non-protein nitrogen showed no significant quantitative changes during the growth period. It was postulated that perhaps a net protein nitrogen increase might be observed, but such was not the case.

All vaccines were soluble from a pH of 14.0 to a pH of 5.56 as determined by back titration with N/10 acetic acid. Cloudiness appeared at about pH 5.5, and precipitation was visible complete at pH 4.9 to 5.0. Molish tests were negative for all vaccines. Protein concentrations and electrophoretic patterns of the various vaccines are shown in figure 4. The three-day cell-free and six-day whole culture vaccines were inadvertantly placed in a freezer following protein determinations, which produced an irreversible gel or thawing; thus, electrophoretic patterns could not be obtained.

Protein concentrations expressed as mg/ml are shown in figure 4. The three-day cell-free culture vaccine and the six-day whole culture vaccine concentrations (not shown) were 11.32 mg/ml and 15.39 mg/ml respectively. Non-protein nitrogen levels of the raw vaccines ranged between 10 and 90 micrograms per ml. All cell-free culture vaccines appeared to have lower protein concentrations than the whole culture vaccines. This would be expected

due to the removal of the protein containing Brucella cells. The reason for the low protein concentration in the six-day cell-free culture was not determined.

Paper electrophoretic studies on the whole culture vaccines (shown in figure 4) indicated that there were two major components, a slightly mobile fraction and a highly mobile fraction. The former fraction migrated in a manner similar to serum globulin, and the latter migrated in a manner somewhat similar to serum albumin. The less mobile fraction increased from 13 per cent in the uninoculated control vaccine to 41.7 per cent in the 13-day whole culture vaccine, or an increase of 28.7 per cent during the 13-day growth period. The cell-free culture vaccines, on the other hand, showed an increase of only 11 per cent in the less mobile fraction over the 13-day growth period. The less mobile fraction was undoubtedly due to some component or components of the Brucella cells, since their removal decreased its concentration by more than half. Titer responses to both vaccines, shown in table 6 and table 7, indicated that the removal of this fraction also removed the majority of agglutinogens from the vaccine. The removal of the intact organisms did not, however, remove all of the agglutinogenic material present. This was probably due to the liberation of agglutinogenic

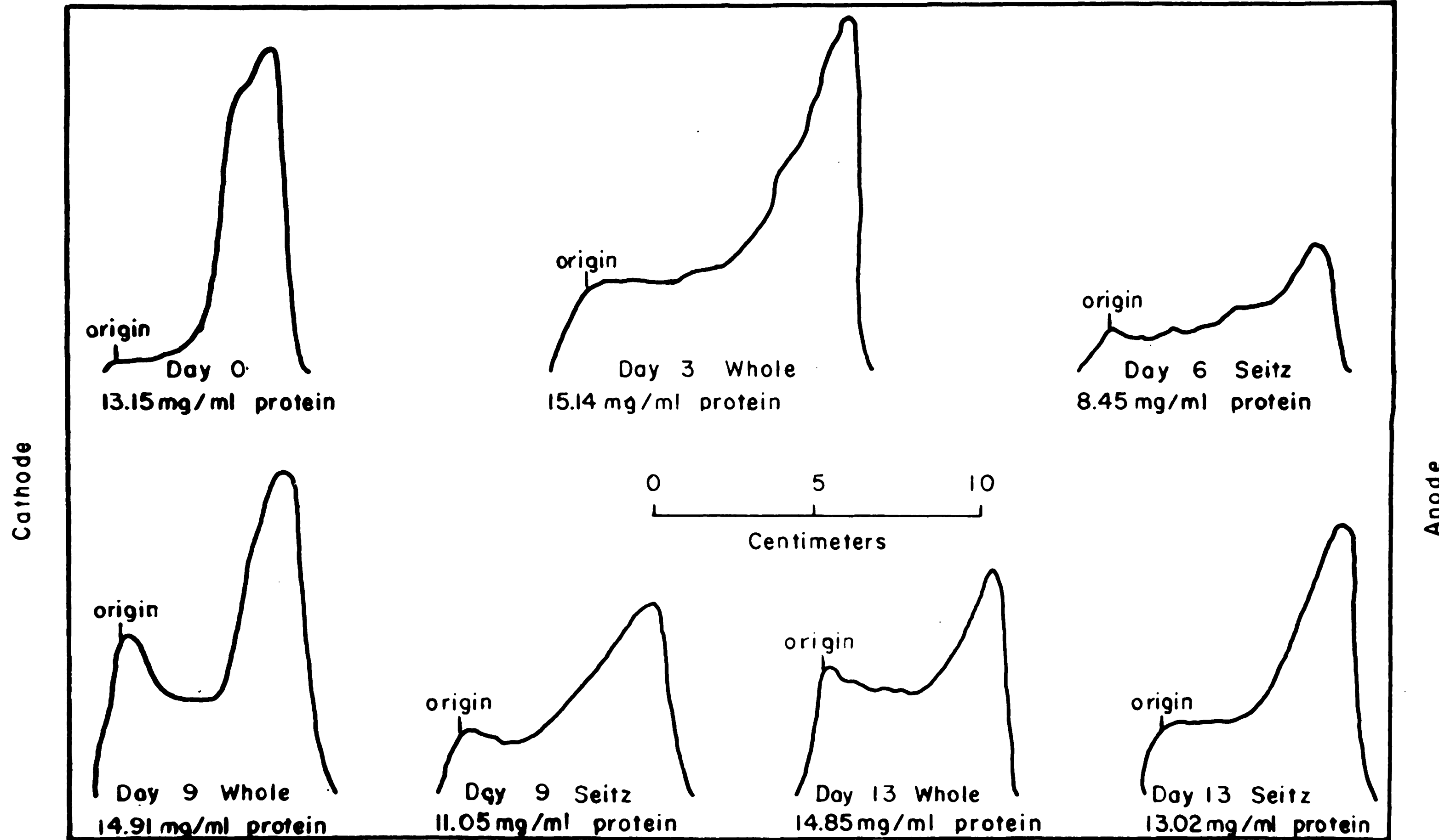

Figure 4. Paper electrophoretic patterns of whole and cell-free culture acid-heat extracted <u>Brucella abortus</u> vaccines prepared at intervals during the 13-day growth period.

TABLE 5

Protocol for Experiment IV. Box titration employing varying amounts of vaccine against varying challenge doses with four guinea pigs per letter group.

Viable brucellae per challenge dose*	Amount of vaccine ml				
		Whole culture		Cell-free culture	
	None	0.25	0.75	0.25	0.75
5.44×10^8	A	E	I	M	Q
5.44×10^7	B	F	J	N	R
5.44×10^6	C	G	K	O	S
5.44×10^5	D	H	L	P	T

* The undiluted challenge material contained 5.44×10^8 viable organisms per ml.

TABLE 6

Brucella agglutinin titers(1) of guinea pigs vaccinated with 13-day acid-heat extracted whole culture and cell-free culture vaccines 18 days post-vaccination (three days pre-challenge).

Guinea pigs per each level	Amount of vaccine ml			
	Whole culture		Cell-free culture	
	0.25	0.75	0.25	0.75
1	- (2)	20	-	-
2	-	80	-	-
3	-	20	-	-
4	-	20	-	-
5	-	20	-	-
Geometric mean	1 (3)	28	1	1

(1) Titer expressed as reciprocal of the dilution.

(2) Negative at 1:20, the lowest test dilution employed.

(3) A geometric mean titer of one indicates all animals were negative at 1:20.

material by bacterial autolysis during the incubation of the Brucella cultures.

Six animals died during the period between vaccination and challenge due to what was apparently a Pasturella multocida pneumonia. One animal died five days following challenge with an acute peritonitis caused apparently by puncture of the liver during the injection of challenge material. These animals are not included in the results.

Brucella recoveries 28 days post-challenge (figure 5) indicated that the ID_{50} of the non-vaccinated controls was 4.19×10^6 viable organisms. In all animals receiving both types of vaccine no infections were produced. Assuming that all animals would have been infected by the next lower dilution of challenge, or 5.44×10^9 viable organisms, both vaccines produced a significant protection of 2.66 logs over the non-vaccinated controls. This indicated that the removal of the Brucella organisms from the culture prior to acid-heat extraction had no apparent effect on the resultant immunogenic property of the vaccine.

The agglutinin response, shown in figure 5, indicated that there was a marked suppression of agglutinins in all animals receiving both types of vaccine. Again as in Experiment II, the higher

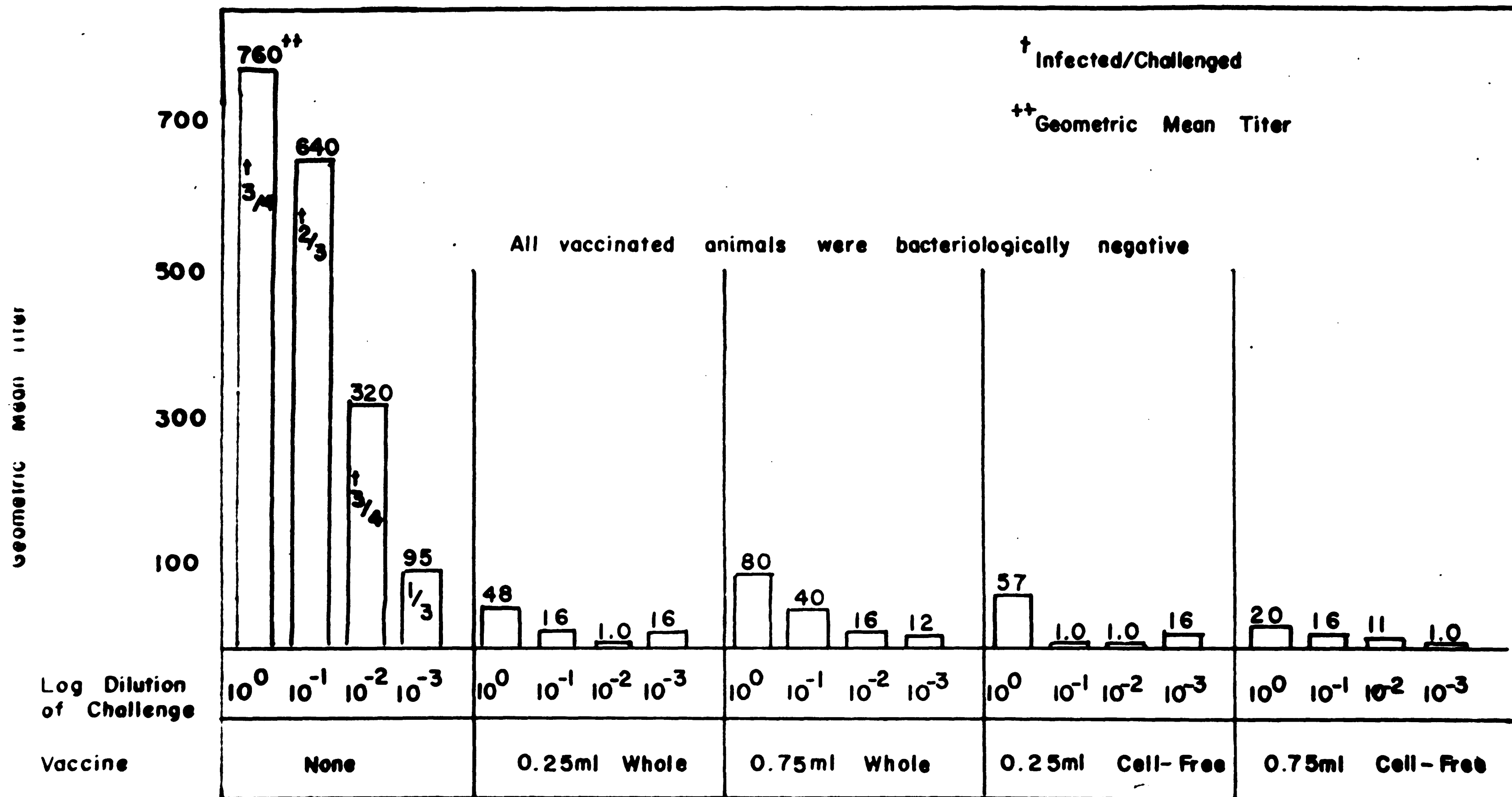

Figure 5. Geometric mean titers and Brucella abortus recoveries 28 days post-challenge from vaccinated and control guinea pigs. Experiment IV. Results obtained from guinea pigs vaccinated with 13-day whole and cell-free culture vaccines with four guinea pigs per group at start.

dosage level of whole culture vaccine produced an agglutinin response in the group receiving the greatest number of challenge organisms. This may have been due to an anamnestic response. This was further evidence to support the observation that the removal of the organisms removed the majority of agglutinogenic material from the vaccine.

The results of this experiment supported the original premise that an effective immunogenic agent, free or essentially free of agglutinogens, might be produced from metabolic by-products.

Experiment V. Response Studies of Acid-Heat Extracted Whole Culture and Cell-Free Culture Vaccines in Calves.

In an attempt to determine the effectiveness of the 13-day whole culture and cell-free culture acid-heat extracted vaccines in cattle, the following work was undertaken.

Procedures: Eight bull calves, varying from six to eight months in age, were isolated and checked for the presence of *Brucella* agglutinins. Serum protein patterns were determined electrophoretically at one and two weeks prior to vaccination. The buffer system used for paper electrophoresis was sodium-barbital-barbituric acid with an ionic strength of 0.053 and a pH of 8.6. Undiluted serum was applied in 0.01 ml amounts to Whatman 3 MM filter paper strips. Serum separations were carried out for five hours at a constant current of 21 ma (Rooney, 1957).

The whole culture and cell-free culture vaccines (Lot 7) prepared in the previous experiment were injected subcutaneously into the cervical region of the calves as shown in table 7. Following vaccination, all animals were bled at weekly intervals for a period of five weeks and agglutinin titers and serum protein patterns were determined. The non-vaccinated control and vaccinated calves were then challenged by subcutaneous injection of 1.0×10^9 viable

Brucella abortus Strain 2308 organisms. _Brucella_ blood recoveries were attempted, serum titers and serum protein patterns were determined daily for seven days. Serum agglutinin titers and serum protein patterns were determined at weekly intervals for an additional two weeks. Post-challenge temperatures were recorded daily for a period of seven days.

Twenty-two days after challenge all animals were sacrificed and _Brucella_ recoveries were attempted from the spleen, a mesenteric lymph node and a testicle from each animal. In further attempts at _Brucella_ recovery from the challenged calves, one ml of a 10 per cent spleen homogenate in basal medium from each calf was injected intraperitoneally into two 250 gm guinea pigs. The guinea pigs were bled for agglutinin titer determination and sacrificed 28 days following injection. Spleens were removed aseptically and _Brucella_ recoveries were attempted.

Results: _Brucella_ recoveries were effected only twice from the blood in the non-vaccinated controls following challenge, as indicated in figure 6. This, in conjunction with the post-challenge titer results, indicated a transient infection only. It was not felt that these recoveries could be used as valid evidence of protection produced in the vaccinated calves. No _Brucella_ recoveries were made from any of the

TABLE 7

Vaccine and post-challenge Brucella agglutinin titers(1) in non-vaccinated and vaccinated calves receiving 13 day whole culture and cell-free culture acid-heat extracted preparations

Vaccine		Calf number	Post-vaccination weeks				Post-challenge days								
Type	Amount ml		1	2	3	4	1	2	3	4	5	6	7	14	21
Challenge control	No vaccine	1	-	-	-	-	-	-	-	40	160	320	640	640	640
		2	-	-	-	-	-	-	-	20	160	640	1280	2560	1280
Whole culture	10(2)	3	160	80	40	-(3)	-	40	40	160	160	320	320	640	1280
		4	40	40	-	-	-	40	40	40	40	40	160	1280	2560
	5	5	160	320	160	-	-	20	40	80	160	640	640	1280	2560
		6	640	80	-	-	-	80	80	320	640	640	640	1280	1280
Cell-free	5	7	40	40	40	-	-	-	-	20	40	80	40	640	80
	10(2)	8	40	20	20	-	-	40	40	40	80	40	-	80	160

(1) Titers expressed as reciprocal of the dilution.
(2) Vaccine given in two 5 ml doses a week apart.
(3) Negative at 1:20, the lowest test dilution employed.

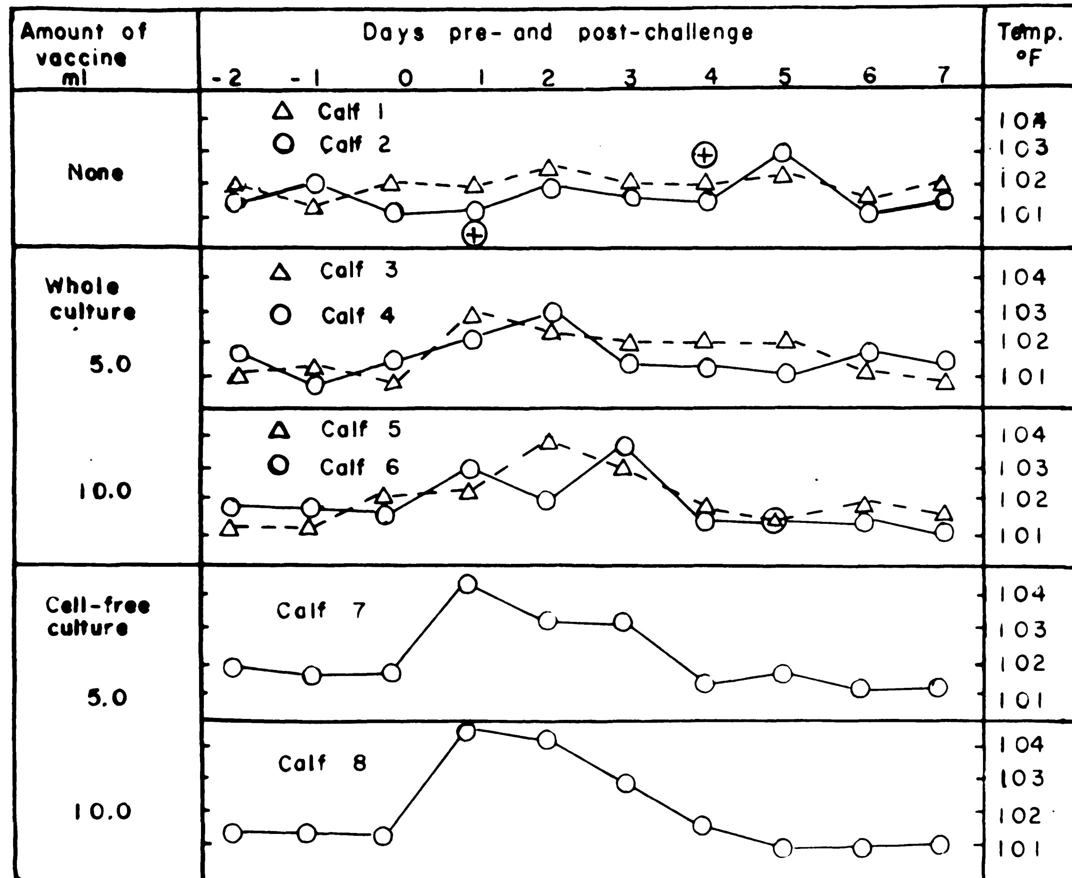

Figure 6. Temperature and Brucella abortus recoveries following challenge of non-vaccinated and vaccinated calves receiving 13-day whole and cell-free culture acid-heat extracted vaccines.

necropsy material 21 days post-challenge, nor were any Brucella recoveries obtained from the guinea pigs through which the calf spleens were passed. None of the guinea pigs demonstrated agglutinin titers 28 days after being injected with the splenic material from the calves. The transient infections in the control animals indicated that, the challenge inoculum was given at too low a level, or that there was a decreased virulence in the challenge organisms due to guinea pig adaptation.

Brucella agglutinins produced by vaccination and challenge are shown in table 7. The lower post-vaccination titers in calves seven and eight indicated that removal of the brucellae from the culture, prior to acid-heat extraction, also removed the majority of the agglutinogens. These same animals also showed a marked agglutinin suppression following challenge when compared to the control calves and those receiving both levels of whole culture vaccine. As may be observed in table 7, only calf eight was serologically negative for brucellosis at the end of the experimental period.

Only the vaccinated calves demonstrated a temperature rise following challenge. It may be noted also that calf eight which showed the greatest titer suppression also demonstrated the highest temperature rise. It could not be determined if the temperature

rises in the vaccinates was a reflection of an immune response. Serum protein patterns, which might have helped to answer this question, showed no significant changes throughout the duration of the experiment.

C. Rabbit Experiments: Selection of Normal Animals and the Effect of Sex and Age on the Immunologic Response to Whole Culture Brucella Vaccines.

In an attempt to determine if there was a direct correlation between agglutinins and gamma globulin produced by the whole culture vaccine, a series of rabbit studies were conducted. Rabbits were chosen for their ease in bleeding and because they are the animal of choice in antibody response studies. The first phase of the problem was to determine the distribution of serum fractions in the normal animal. It became apparent at the outset(of this phase of the problem) that clinically normal rabbits often showed dysproteinemia on paper electrophoretic analysis. This finding and lack of adequate data in the literature necessitated a study of the serum protein patterns of rabbits before gamma globulin response studies could be made.

The major reports that have appeared on the fractionation of serum proteins of domestic animals, using paper electrophoresis, are those of Chopard (1954) and Boguth (1953, 1954). However, only Chopard (1954) and Chiaroni and Nardi (1956) appeared to have utilized this method for the examination of rabbit serum. The former worker briefly mentioned the normal rabbit and based his results on only four fractions and the latter compared

zone and moving boundary results of normal rabbits. There was rather a wide range of values between the two reports and no mention was made of any sex or breed differences.

Experiment VI. Paper Electrophoretic Analysis of Rabbit Serum as an Aid in the Selection of Experimental Rabbits

The purpose of this phase of the problem was to examine the serum protein patterns of clinically healthy rabbits, to establish normal ranges and to observe possible breed and sex differences.

Procedures: Thirty adult rabbits of various breeds, equally divided as to sex were obtained locally. The rabbits were isolated for two weeks in individual cages for observation, and acclimation. Parasitological examinations were also performed.

Blood samples were taken at weekly intervals for four weeks and serum protein pattern determinations were made. The buffer system used was sodium-barbital-barbituric acid, with an ionic strength of 0.075 and a pH of 8.6. Undiluted serum (0.008 ml) was applied to Whatman 3 MM filter paper strips and the separations were carried out for 16 hours at a constant current of 5 ma. Following the end of the four week protein pattern study, the rabbits were sacrificed and histopathological studies were made on necropsy material.

<u>Results</u>: The following normal serum protein percentages were obtained from 12 male and 6 female rabbits: albumin 64.01 ± 1.63 per cent; alpha 1 globulin, 7.21 ± 0.96 per cent; alpha 2 globulin, 5.80 ± 1.05 per cent; beta globulin, 12.77 ± 1.20 per cent; and gamma globulin, 9/91 ± 1.10 per cent. They are the average of three to four blood samples from each rabbit. These values were averaged and the mean value of each fraction was determined and expressed as the percentage of the total protein present (table 10). The values for the abnormal rabbits (table 10) were computed similarly.

Representative serum protein patterns of normal and abnormal rabbits are shown in (figure 7). The pattern of normal rabbits is shown as a composite pattern from one bleeding of the 18 normal animals.

Migration values of normal animals are shown in table 9. Since the migration is constant within each run, but not from run to run, a C/AF value was used to allow comparison of migration values. The equation used cancels inherent variables between runs, such as different batches of buffer, differences in paper strips and line current fluctuations. The formula is given below.

$$C/AF = \frac{\text{Distance of component migration (center of band) from origin}}{\text{Distance of albumin front from origin}}$$

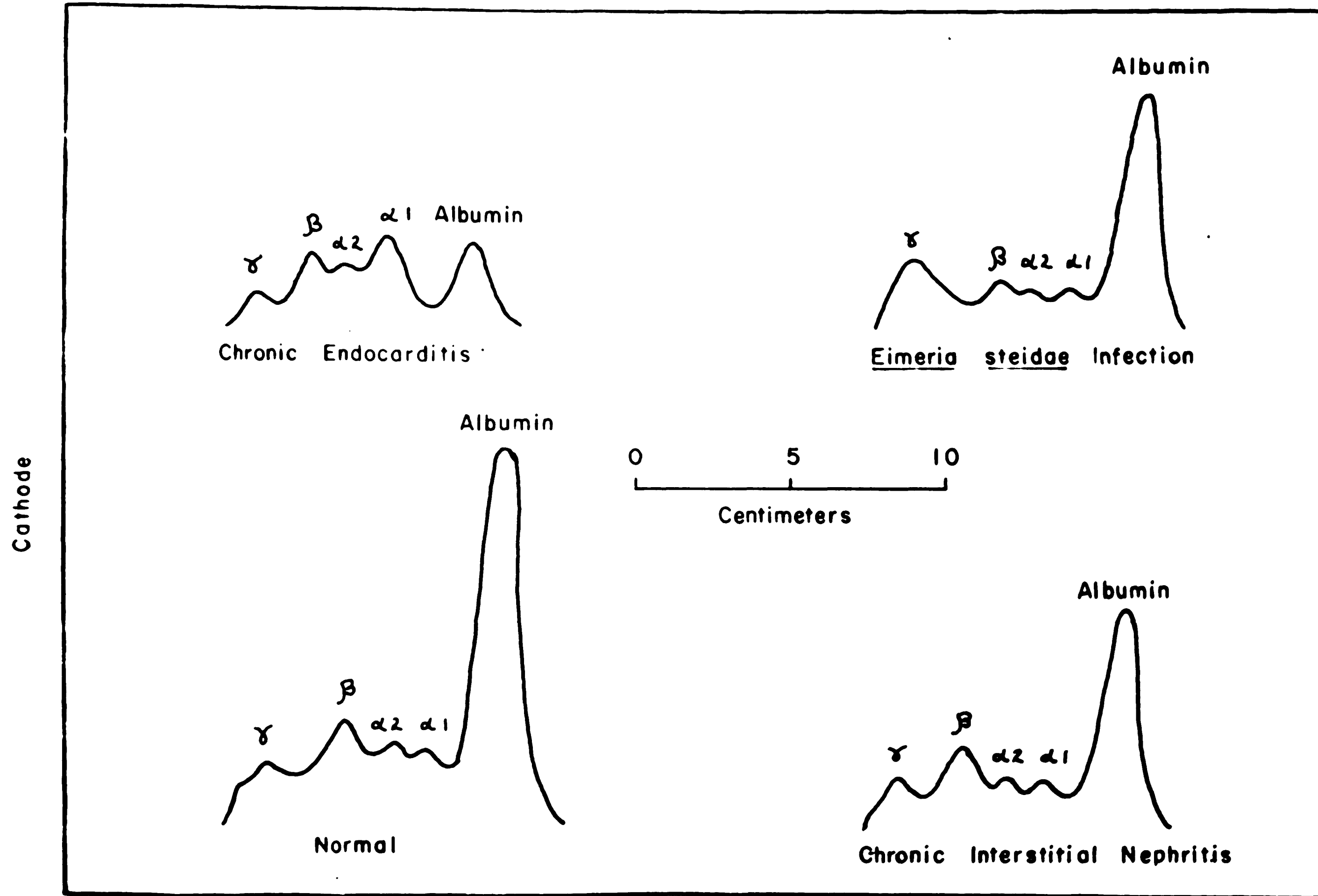

Figure 7. Paper electrophoretic serum protein patterns of normal and abnormal rabbits.

The C/AF values were calculated for those components migrating toward the anode. Gamma globulin migration was calculated directly in a direction toward the cathode. In the normal group, there was greater variation among rabbits than between consecutive samples from a single rabbit. Reproducibility was determined by using the same serum on different runs. A range of two to three per cent in albumin, and a correspondingly smaller range in the globulins, between different analyses was found.

Breed and sex appeared to have no influence on the serum patterns or on the migration values (table 8). Insufficient numbers of animals were used to determine any breed differences.

Chiarioni and Nardi (1956) reported the following results for paper electrophoresis of rabbit serum: albumin, 57.21 per cent; alpha 1 globulin, 6.04 per cent; alpha 2 globulin 5.72 per cent; beta globulin; 11.25 per cent; and gamma 1 and gamma 2, 16.96 per cent. In this study, none of the rabbits considered normal had an albumin level as low or a gamma globulin level as high as those reported by these authors. Gamma globulin has been reported as a single fraction in this study. With these exceptions, the results agreed with those of Chiarioni and Nardi (1956) and

TABLE 8

Serum protein values of normal rabbits according to breed and sex.

Breed	Sex	Mean per cent of total serum protein				
		Albumin	Alpha 1	Alpha 2	Beta	Gamma
Albino	F	65.70	7.65	5.86	10.50	10.28
Black Giant Cross	F	63.70	7.93	5.59	14.08	10.53
Black Giant Cross	F	63.30	5.45	6.46	10.80	13.10
Chinchilla	F	63.73	7.57	5.34	13.03	10.27
New Zealand Red	F	65.40	8.11	5.66	11.58	9.02
New Zealand Red	F	62.20	8.35	4.00	14.70	10.06
Albino	M	63.63	7.61	5.76	12.46	10.17
Albino	M	63.41	8.08	5.81	13.97	8.83
Albino	M	62.00	6.67	7.97	13.47	9.87
Albino	M	66.00	5.72	5.86	12.60	9.80
Albino	M	65.60	7.48	4.72	12.20	10.00
Black Giant Cross	M	62.66	7.29	7.18	12.56	9.75
Black Giant Cross	M	61.43	8.36	7.61	14.26	8.34
Black Giant Cross	M	65.15	6.90	5.92	13.87	7.72
Black Giant Cross	M	64.70	7.58	4.92	12.30	10.12
Chinchilla	M	67.30	6.89	4.57	11.14	10.03
Chinchilla	M	63.36	5.12	6.68	12.83	11.75
Chinchilla	M	65.70	6.97	4.66	13.61	8.77

Chopard (1954); who found 62.5 per cent albumin, 10.7 per cent alpha, 14.8 per cent beta, and 12 per cent gamma globulin.

Boundary electrophoretic values for the serum components of rabbits (Spector, 1956) agreed within one or two per cent of the results obtained here. A theta fraction reported by Spector was not apparent using paper, and in this work may be represented in the total gamma globulin.

The five animals with kidney lesions (table 10) showed a marked decrease in albumin due to loss in the urine (as demonstrated by the Robert's test). These animals showed elevated beta and gamma globulin levels which represent a ratio change rather than an increased globulin production. A composite pattern of these animals is shown in figure 7. It was interesting to note the pronounced difference between alpha globulins in amyloid nephrosis and interstitial nephritis (table 10). These indicated that the tissue destruction causing amyloid nephrosis was detectable by this method; thus, distinguishing amyloid nephrosis from interstitial nephritis.

The animals with minimal portal round cell infiltration in the liver (table 10) had albumin levels in the lower normal range and a marked increase in gamma globulin. The other globulins

Table 9

Serum protein migration of normal rabbits expressed as a C/AF ratio (1)

Sex	No.	C/AF ratio of serum protein components				
		Albumin	Alpha 1	Alpha 2	Beta	Gamma (2)
F	6	.906 ± .001	.596 ± .004	.467 ± .009	.328 ± .006	-501 ± .006
M	12	.907 ± .006	.609 ± .010	.478 ± .009	.334 ± .004	-501 ± .005
Both		.906 ± .004	.604 ± .008	.474 ± .009	.332 ± .005	-501 ± .005

(1) C/AF Ratio = $\frac{\text{Distance of component migration (center of band) from origin}}{\text{Distance of albumin front from origin}}$

(2) Measured directly from origin to center of component band.

TABLE 10

Serum protein values of normal and abnormal rabbits

Necropsy Findings	No. Animals	Mean per cent total serum protein				
		Albumin	Alpha 1	Alpha 2	Beta	Gamma
Normal	18	64.01	7.21	5.80	12.77	9.91
Nephritis (interstitial)	4	48.4	8.77	6.75	17.5	18.1
Amyloid nephrosis	1	42.76	13.36	8.61	14.53	18.64
Bile duct infected with *Eimeria stiedae*	1	40.9	7.75	6.97	13.98	30.0
Liver: minimal portal round cell infiltration	2	60.62	6.28	4.64	11.26	16.96
Liver: hemorrhage and necrosis Cecum: necrotic cecitis with lymphoid proliferation	1	47.66	9.18	8.59	15.73	18.90
Endocarditis (chronic)	1	22.80	31.60	12.53	21.70	11.33
Uterine cyst - left horn 195 cc. proteinaceous fluid removed	1	45.75	11.67	10.10	12.50	19.40

showed little or no change from those animals considered normal. It was not clear if the high gamma globulin levels in these animals were the result of response to prior infection with resulting round cell infiltration (Thorebecke and Keuning, 1956). The chronic endocarditis pattern (figure 7) was included to show the effect of liver damage as evidenced by the decreased albumin and beta globulin levels. It also demonstrated marked tissue degeneration as evidenced by the increase in the alpha globulins. This condition also appeared to alter the migration C/AF ratios of the different serum fractions. Since the results are based on one case, it was not possible to determine if this was an artifact or actually indicated the presence of altered proteins.

Eimeria stiedae infection appeared to decrease the albumin level, probably by causing damage in the liver. The remaining globulins with the exception of gamma globulin showed no change, There was a three-fold increase in gamma globulin, which indicated a marked immunogenic response. The pattern shown was a composite scan of an additional 14 rabbits in which Eimeria stiedae infection was established by fecal examination (figure 7). Individual values for these animals are shown in table 11 and were those obtained following sulfaquinoxiline treatment. The findings were consistent

TABLE 11

Serum protein values of six to eight month old rabbits infected with *Eimeria stiedae*

Sex	Per cent total serum protein				
	Albumin	Alpha 1	Alpha 2	Beta	Gamma
F	45.60	8.20	5.85	15.75	24.60
F	43.40	6.28	4.40	13.20	32.60
F	35.9	6.12	5.31	17.5	35.00
F	45.0	9.75	7.50	18.0	19.50
F	49.50	7.32	6.50	17.05	19.55
F	44.00	8.68	5.80	15.22	26.10
F	37.2	9.3	5.59	19.50	28.30
F	42.0	8.56	6.44	15.0	27.80
M	53.5	9.36	4.92	11.7	25.60
M	53.8	8.02	5.05	8.78	24.00
M	44.7	8.7	4.95	14.25	27.3
M	53.0	7.80	5.60	11.20	22.40
M	41.0	7.05	3.84	17.3	38.20
M	30.2	10.8	5.88	14.3	38.60

with the varying degrees of liver damage found at necropsy.

The remaining animals (table 10) with more than one pathological change showed marked dysprotenemia, but no attempt was made to correlate the patterns with any one specific lesion.

Experiment VII. The Effect of Sex and Age on the Brucella Agglutinin Titer Response and Serum Protein Changes in Rabbits Vaccinated with Whole Culture Vaccines.

The purpose of this portion of the investigation was two-fold: to ascertain if sex and age were determining factors in the immunologic response to the vaccine if the agglutinin response could be directly correlated with any serum protein changes. This experiment was conducted in three trials using three lots of vaccine.

Procedures, Trial 1: Three male and three female rabbits, one-year-old, were separated individually in wire cages. *Brucella* agglutinins, and base line serum protein patterns were determined for a period of three weeks. All animals then received approximately 20 mg of protein from Lot 3 raw vaccine injected subcutaneously in the cervical region. Agglutinin titers and serum protein patterns were determined for five weeks following vaccination, or until the titers had leveled off.

Trial 2: Six male and six female rabbits, six-months-old, were separated in wire cages and treated similarly to trial one prior to vaccination. All animals received 20 mg of protein material from Lot 4 raw vaccine. Agglutinin titers and serum protein patterns were determined for five weeks following vaccination.

Trial 3: Twelve male and twelve female rabbits were divided

into groups of three as follows: three males and females one-year-old; three males and females six-months-old, three castrated males and three ovario-hysterectomized females one-year-old and three castrated males and three ovario-hysterectomized females six-months-old. Untreated animals were handled similarly to trials one and two with the exception that 30 mg of proteinaceous material from Lot 7 vaccine was employed. Serological titers and serum gamma globulin levels were determined for an eight week period, due to the larger amount of vaccine given.

Castrated animals received 0.1 mg of Estradiol cyclopentyl propionate (ECP) per pound of body weight. Ovario-hysterectomized animals received 0.4 mg of aqueous Testosterone per pound of body weight. These hormones were injected intramuscularly from three weeks pre-vaccination to three weeks post-vaccination at weekly intervals to determine if a reversal of sex would lead to a reversal of the type of agglutinin response. The hormones were obtained from the Upjohn Company, Kalamazoo, Michigan.

Results: Serum gamma globulin changes following vaccination are shown in table 12. It may be observed that the older rabbits

TABLE 12

The effect of acid-heat extracted 13-day whole culture vaccines on the serum gamma globulin level in male and female rabbits.

Trial	Age months	Sex	Number* of animals	Mean per cent increase	Post-Vaccination days
1	12	Male	3	22.31	22
		Female	3	9.59	21
2	6	Male	6	6.64	21
		Female	6	5.48	16
3	12	Male	3	7.56	16
		Female	4	6.43	16
	6	Male	1	2.20	30
		Female	3	5.30	17
	12	Castrated	2	5.40	21
		Spayed	1	6.90	21
	6	Castrated	2	3.66	34
		Spayed	3	5.09	14

* Animals showing abnormalities in serum protein levels and on necropsy examination are not included in gamma globulin response data.

responded with greater increases than the younger rabbits. The males showed a greater increase than females. The hormone treated castrated and ovario-hysterectomized animals appeared to show a reversal of gamma globulin response in the two sexes.

The peak gamma globulin response followed the maximum agglutinin response by seven to twenty-three days. This indicated that the rise in gamma globulin could not be attributed solely to the agglutinin response. This may have been due to a secondary response, the nature of which was not determined.

Interstitial nephritis became apparent in several rabbits in trial three following the initial base line studies. These animals were not included in the gamma globulin response studies due to the false high gamma globulin levels which occurred as a result of the ratio change in the serum protein fractions. This condition was without effect on the agglutinin titers, therefore, these animals were retained in this part of the study.

The Brucella agglutinin titer decline of untreated rabbits in all three trials (figures 9-12) indicated that the females responded with slightly higher initial titers than males. The agglutinin level decline in females occurred at a significantly lower rate than in the males. Age appeared to play no role in the initial agglutinin

agglutinin response, or in the subsequent agglutinin decline. The overall regression of titer decline for both males and females, without regard to age or dosage of vaccine, is shown in figure 14. The slopes indicated that the titer in the males declined one-half again more rapidly than the females. However, when both females and males in trial three were corrected to five weeks for dosage level difference, females showed no change in rate while the rate at decline in the males increased 20 per cent. These results indicated that for similar vaccine dosage levels the rate of agglutinin decline in males probably would be double that of the females.

The analyses of variance (Kramer, 1959; Snedecor, 1946; Fisher and Yates, 1949) of the untreated rabbits (table 13 to 16) indicated that the most important factor in the rate of decline was due to sex. The statistically significant variations in animals within sex and sex by weeks are not practically significant. A serological titer variation of one-half tube in dilution appears statistically significant when the error term is increased. This difference has no serological significance and can be attributed to pipetting errors and differences in antigens. Any serological titer would be expected to decrease as time increased; therefore,

this variable played no role in the effect of sex on agglutinin response.

Attempts to reverse the sex of the animals appeared successful in the six-month-old group. The castrated males showed a higher initial titer over the first five weeks (figure 13). However, the titer began to drop rapidly two weeks following cessation of ECP. The six-month-old females showed little effect from ovario-hysterectomy and testosterone treatment as compared to the normal six-month-old females. From a serological stand-point there was little or no serological difference in both treatments when compared to the normal females of the same age.

In the year-old castrated and, ovario-hysterectomized animals (figure 12) there appeared to be a reversal of titer decline up to the cessation of hormone treatment. There was a subsequent leveling off of male titers with a continued drop in the females following the removal of hormone treatment. In this group the males are serologically identical to the normal females of a similar age. The ovario-hysterectomized animals showed a pattern similar to normal males for six of the eight weeks of the experiment.

Analysis of variance of both age groups (tables 17 and 18) showed that sex played little or no role in the titer decline in the

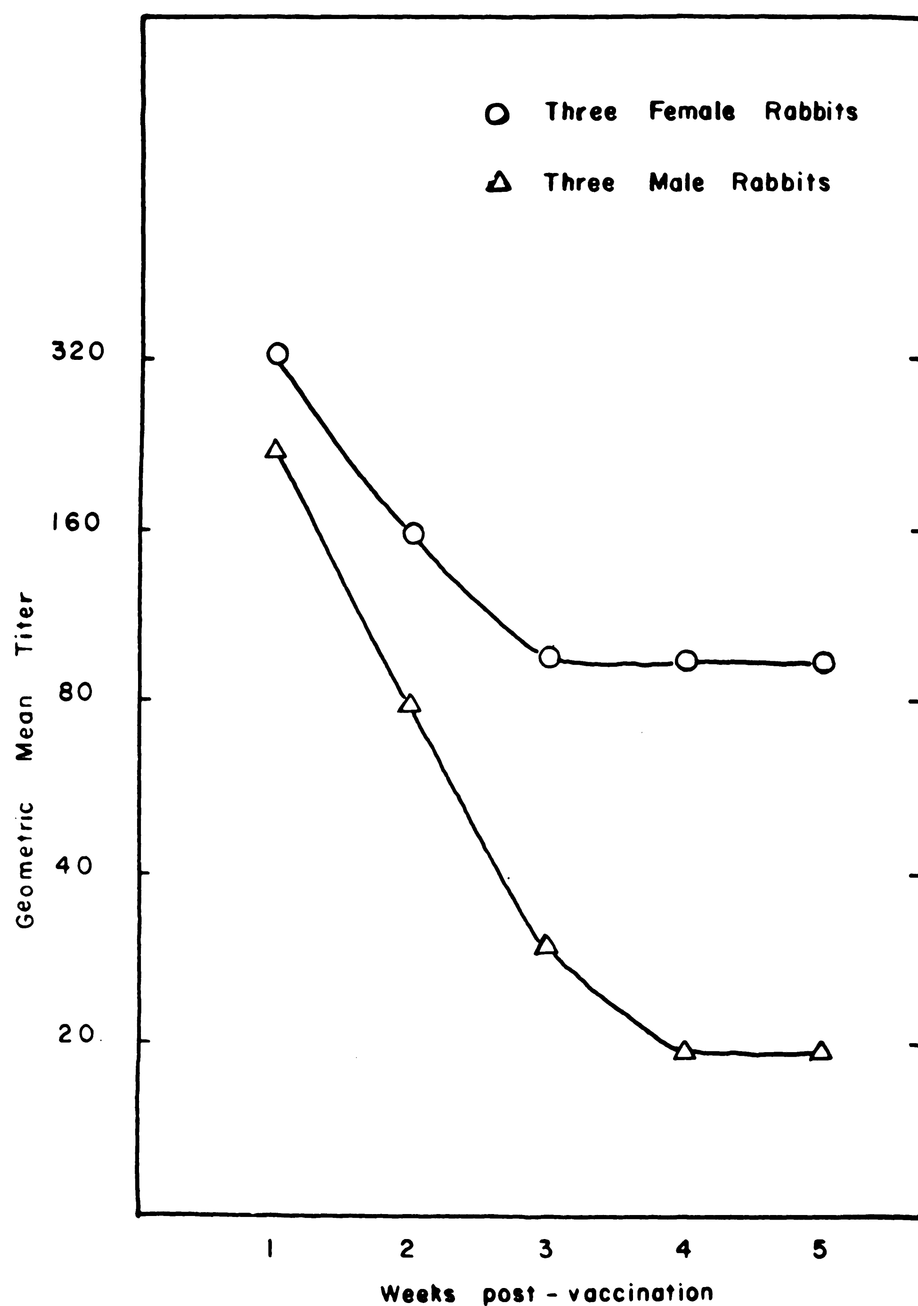

Figure 8. Agglutinin titer decline in male and female rabbits one-year-old vaccinated with approximately 20 mg protein of Lot 3 vaccine without adjuvant.

TABLE 13

Analysis of variance of *Brucella* agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in rabbits one-year-old vaccinated with 20 mg protein of Lot 3 vaccine.

Source	Degrees of freedom	Sums of squares	Means of sums of squares	F value from data	F at 95% level
Animals w/in sex	4	1.8600	.4850	1.264	3.01
Sex	1	32.0333	32.0333	83.459	4.49
Weeks	4	41.1300	10.2825	29.977	3.01
Sex x weeks	4	1.1900	.2975	.775	3.01
Error	16	6.1400	.3837		
Total	29	82.3533			

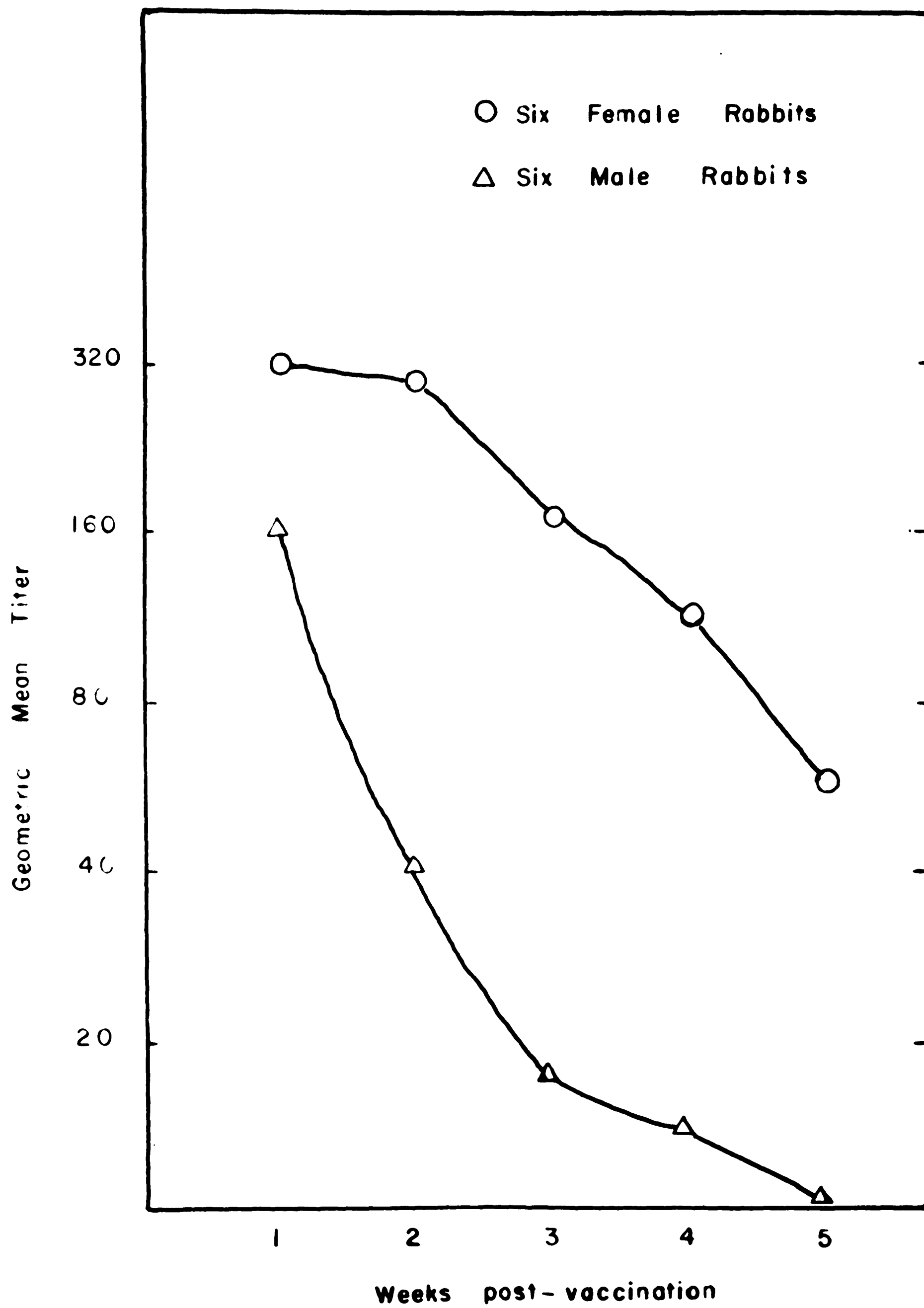

Figure 9. Agglutinin titer decline in male and female rabbits six-months-old vaccinated with 20 mg protein of Lot 4 vaccine without adjuvant.

TABLE 14

Analysis of variance of <u>Brucella</u> agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in rabbits six-months-old vaccinated with 20 mg of Lot 4 vaccine

Source	Degrees of freedom	Sums of squares	Means of sums of squares	F value from data	F at 95% level
Animals w/in sex	10	15.8333	1.5833	3.166	2.09
Sex	1	101.4000	101.4000	202.8	4.03
Weeks	4	81.0670	20.2667	40.533	2.61
Sex x weeks	4	14.2666	3.5666	7.133	2.61
Error	40	20.0000	.5000		
Total	59	232.5669			

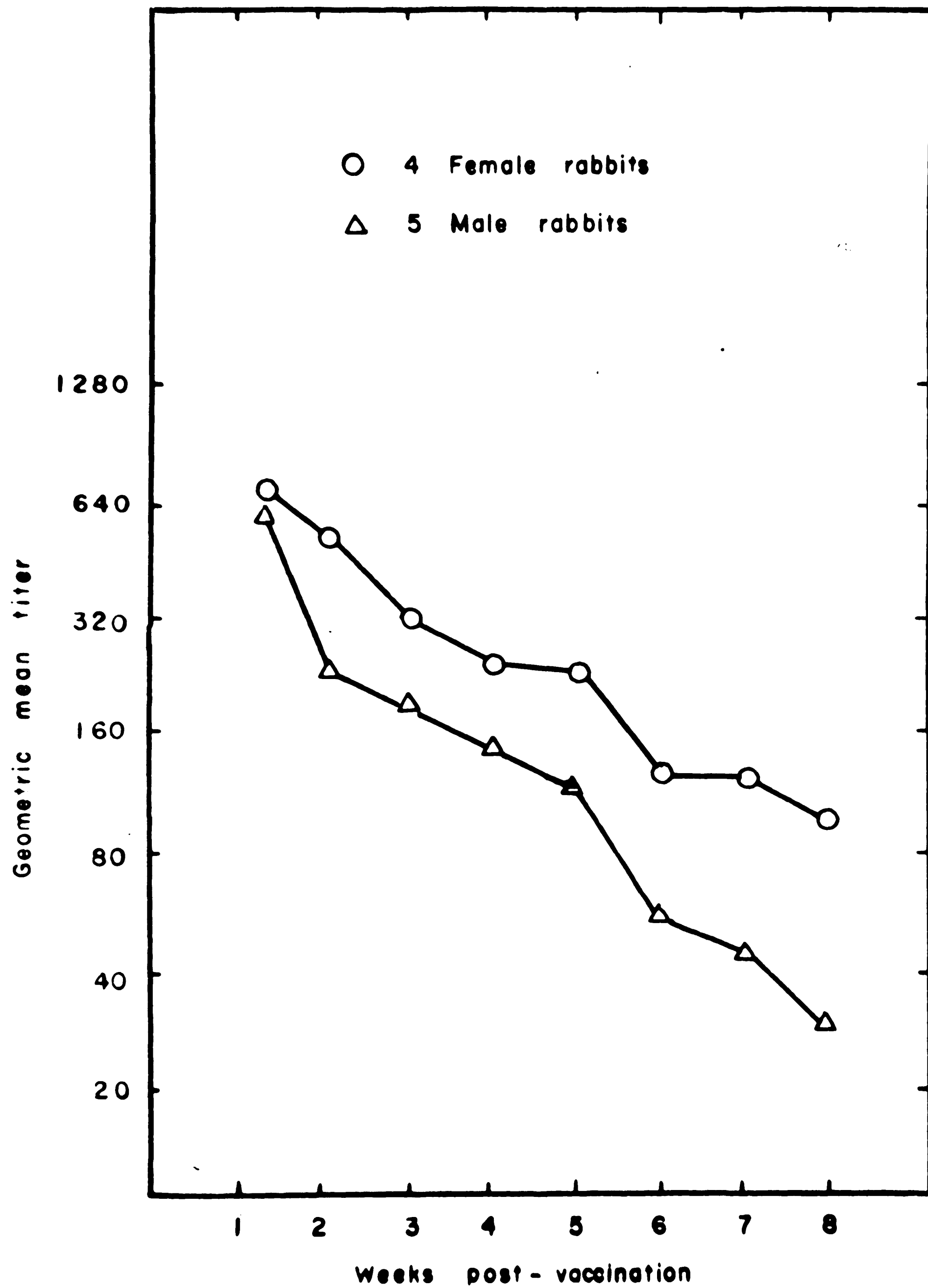

Figure 10. Agglutinin titer decline in male and female rabbits one-year-old vaccinated with 30 mg protein of Lot 7 vaccine without adjuvant.

TABLE 15

Analysis of variance of Brucella agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in rabbits one-year-old vaccinated with 30 mg protein of Lot 7 vaccine

Source	Degrees of freedom	Sums of squares	Means of sums of squares	F value from data	F at 95% level
Animals w/in sex	7	17.1000	2.4285	7.0005	2.34
Sex	1	14.4000	14.4000	41.510	4.00
Weeks	7	98.6666	14.0952	40.629	2.34
Sex x weeks	7	3.9000	.5571	1.603	2.34
Error	49	17.000	.3469		
Total	71	151.0666			

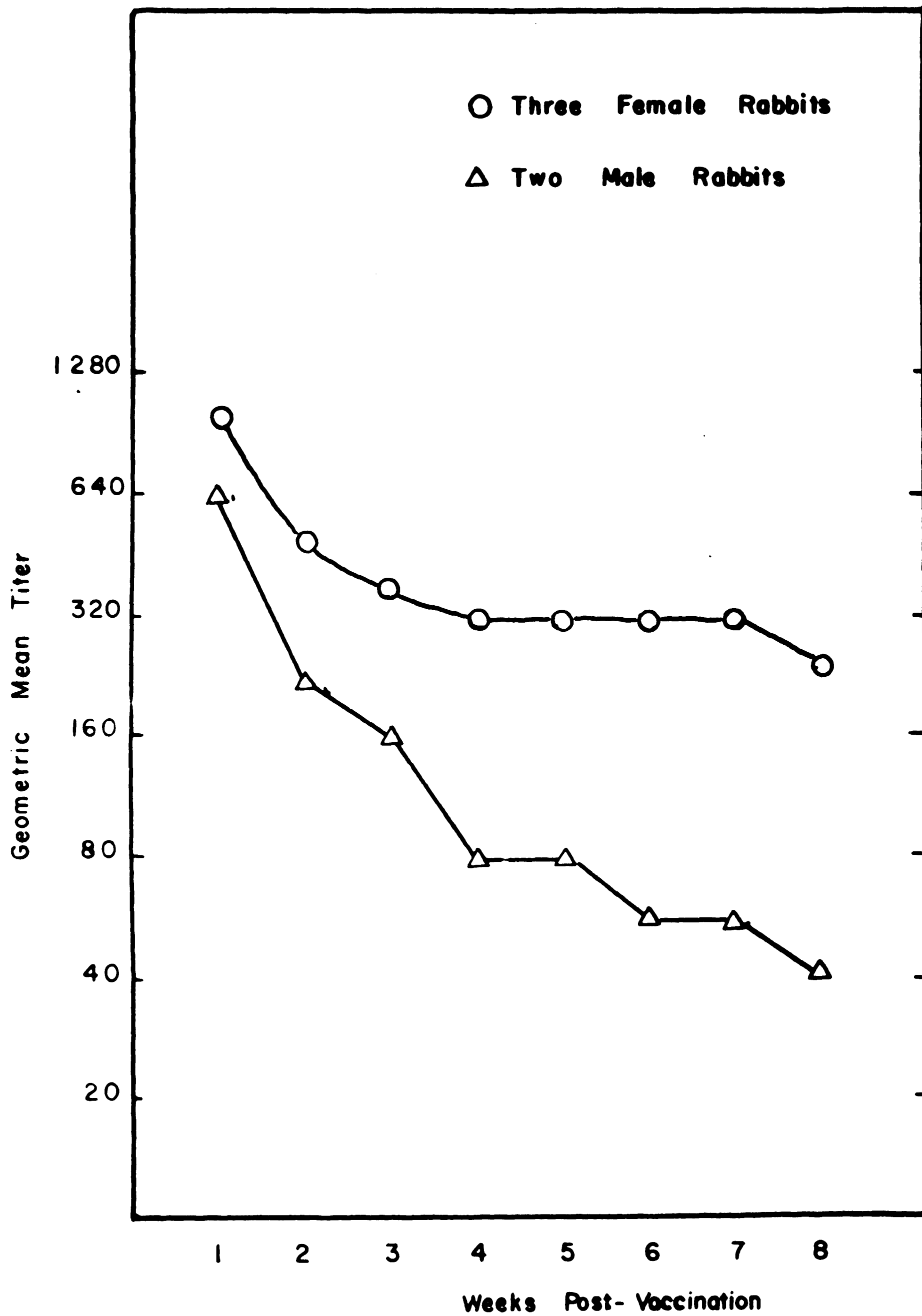

Figure 11. Agglutinin titer decline in male and female rabbits six-months-old vaccinated with 30 mg protein of Lot 7 vaccine without adjuvant.

TABLE 16

Analysis of variance of *Brucella* agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in rabbits six-months-old vaccinated with 30 mg protein of Lot 7 vaccine

Source	Degrees of freedom	Sums of squares	Means of sums of squares	F value from data	F at 95% level
Animals w/in sex	3	9.7460	3.2487	6.547	2.84
Sex	1	31.0040	31.0040	74.574	4.08
Weeks	7	28.8000	28.8000	8.291	2.34
Sex x weeks	7	2.0290	.2896	.583	2.34
Error	21	10.4230	.4962		
Total	39				

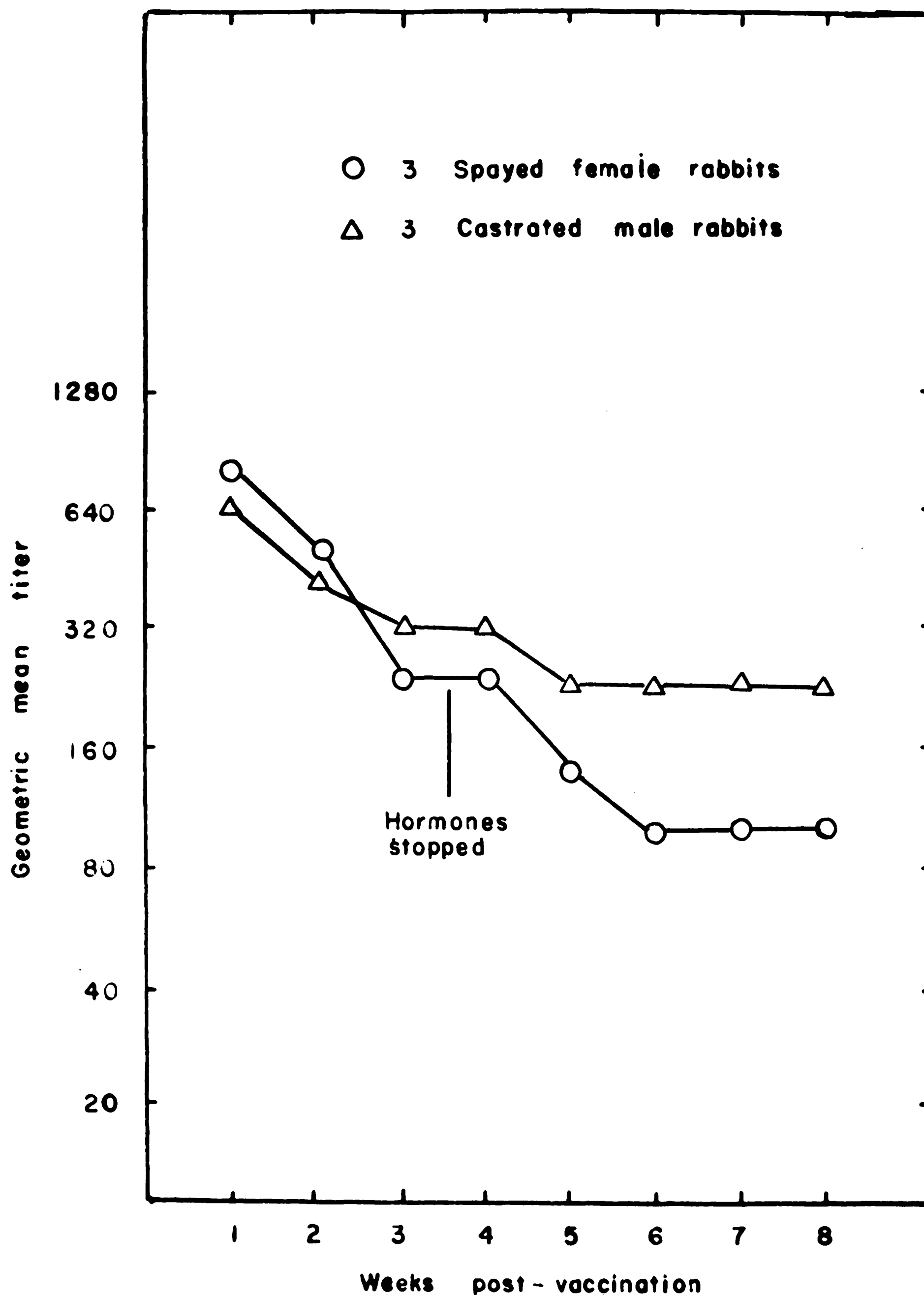

Figure 12. Agglutinin titer decline in one-year-old castrated males treated with 0.1 mg ECP/lb of body weight and ovario-hysterectomized females treated with 0.4 mg aqueous testosterone/lb of body weight vaccinated with 30 mg protein of Lot 7 vaccine without adjuvant.

TABLE 17

Analysis of variance of Brucella agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in castrated[1] and ovario-hysterectomized[2] rabbits six-months-old vaccinated with 30 mg protein of Lot 7 vaccine

Source	Degrees of freedom	Sums of squares	Means of sums of squares	F value from data	F at 95% level
Animals w/in sex	4	10.9590	2.7398	10.594	2.71
Sex	1	.6450	.6450	2.494	4.20
Weeks	7	74.8123	10.6875	41.715	2.29
Sex x weeks	7	8.5217	1.2174	4.746	2.29
Error	28	7.0410	.2586		
Total	47				

(1) Castrated animals received 0.1 mg ECP/lb body weight weekly from three weeks prior to vaccination to three weeks post-vaccination.

(2) Ovario-hysterectomized animals received 0.4 mg aqueous testosterone weekly from three weeks prior to vaccination to three weeks post-vaccination.

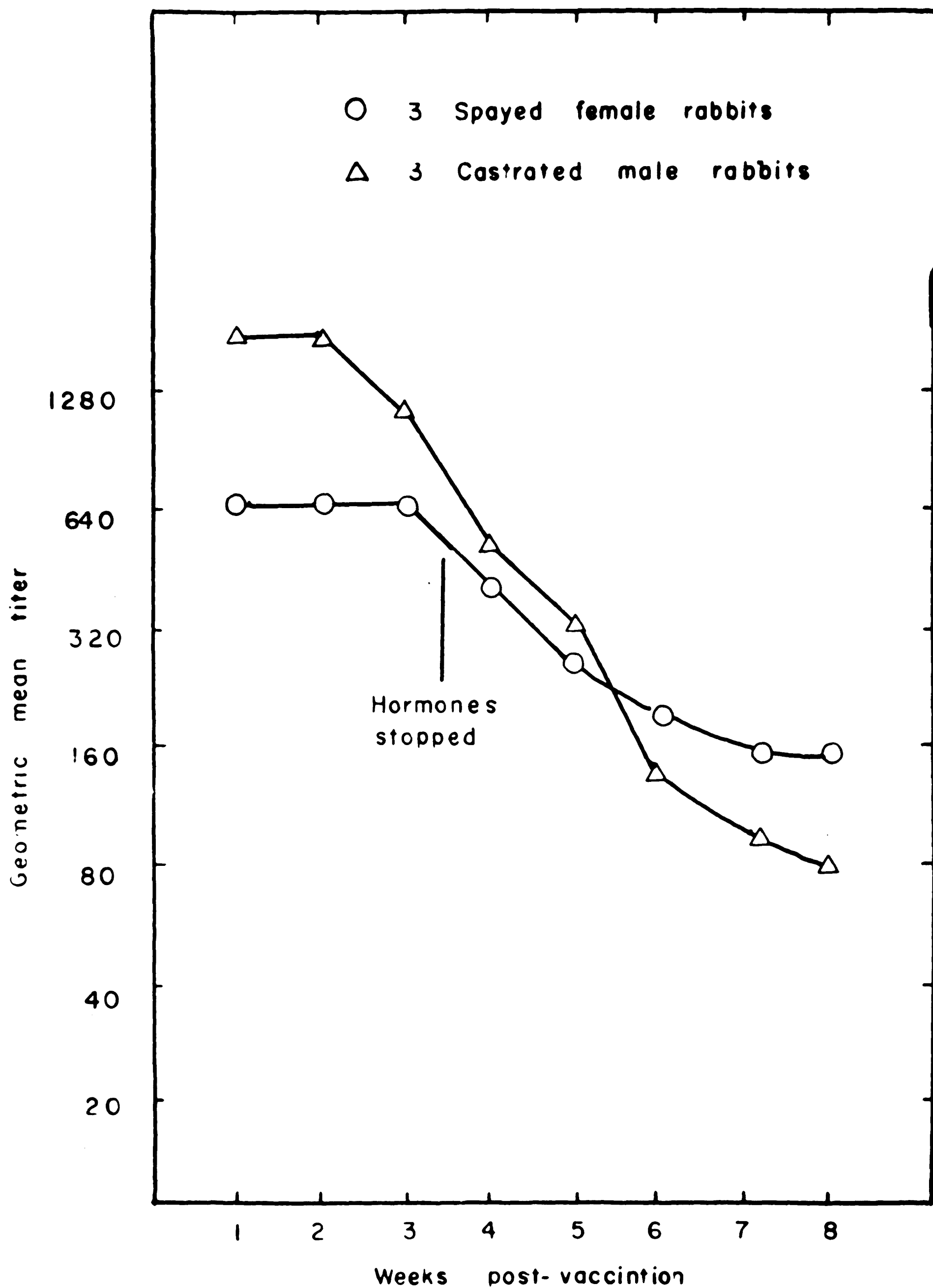

Figure 13. Agglutinin titer decline in six-month-old castrated males treated with 0.1 mg ECP/lb body weight and ovario-hysterectomized females treated with 0.4 mg aqueous testosterone/lb of body weight vaccinated with 30 mg of Lot 7 vaccine without adjuvant.

TABLE 18

Analysis of variance of *Brucella* agglutinin titer decline for differences due to sex, time, animals within sex and sex by time in castrated(1) and ovario-hysterectomized(2) rabbits one-year-old vaccinated with 30 mg protein of Lot 7 vaccine

Source	Degrees of freedom	Sums of squares	Means of sums of squares	F value from data	F at 95% level
Animals w/in sex	3	18.6670	6.2223	7.609	3.07
Sex	1	3.9833	3.9833	4.873	4.32
Weeks	7	29.1000	4.1571	5.086	2.48
Sex x weeks	7	2.9830	4.2614	5.213	2.48
Error	21	17.167	.8174		
Total	39				

(1) Castrated animals received 0.1 mg ECP/lb body weight weekly from three weeks prior to vaccination to three weeks post-vaccination.
(2) Ovario-hysterectomized animals received 0.4 mg of aqueous testosterone weekly from three weeks prior to three weeks post-vaccination.

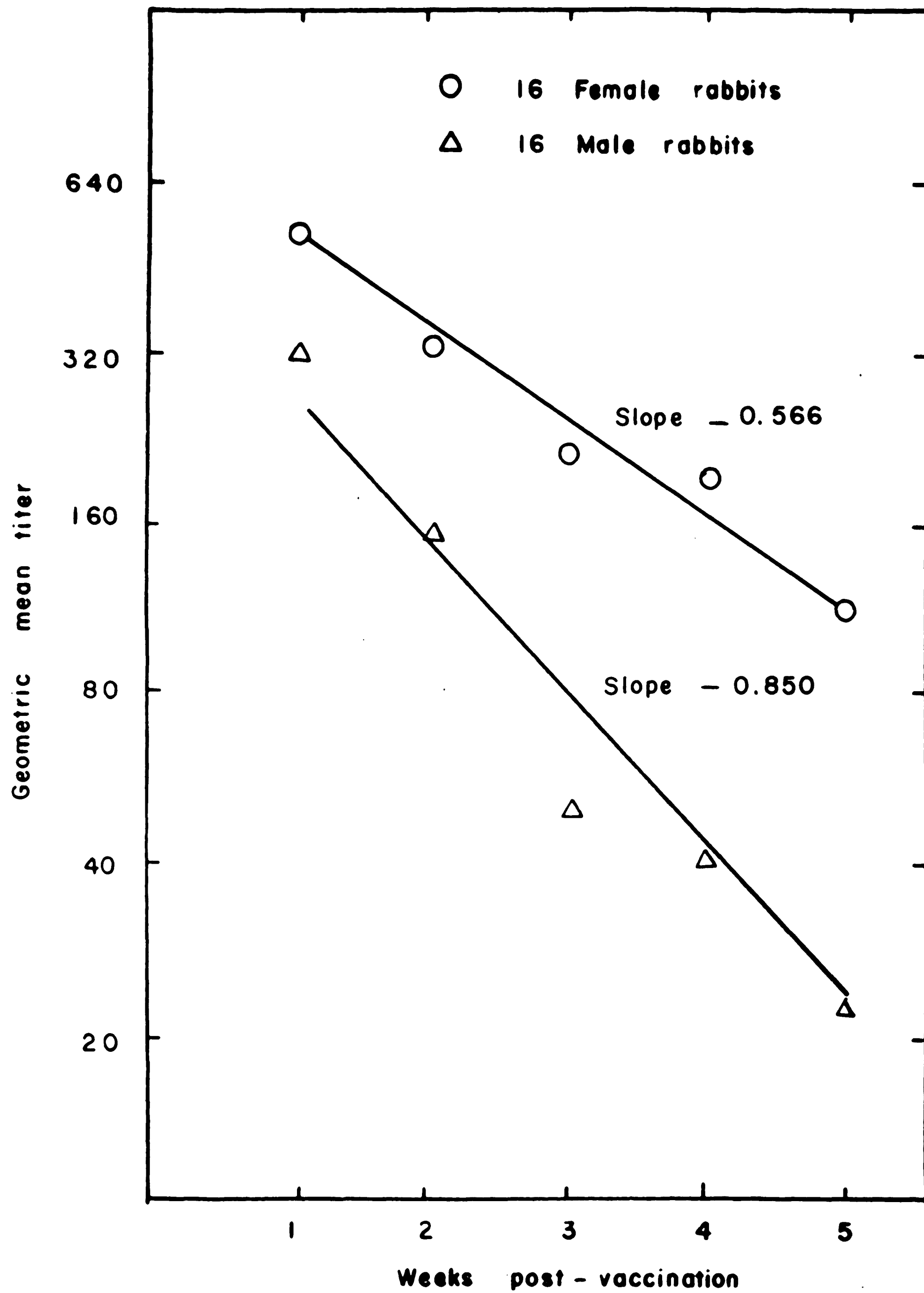

Figure 14. Linear regression of the overall agglutinin titer decline in male and female rabbits without regard to age, dosage or vaccine lot.

castrated and ovario-hysterectomized animals. Insufficient animals were used to determine if age was a factor in the castrated and ovario-hysterectomized animals. No definite conclusions may be drawn concerning the role of age in these treated animals. It appeared, however, that age might be a possible factor and should be taken into consideration in future work with treated animals.

VI. DISCUSSION

A. Immunogenic Studies

No comparison was possible between the vaccine used in Experiment I and the types prepared in subsequent experiments due to the differences in media used. In addition, the use of adult cattle for the study of agglutinin response does not warrant comparison to the calf study; since adult animals react with a stronger agglutinin response.

It was apparent from the experimental data that both the agglutinogenic and immunogenic material were associated with the metabolic by-products rather than the *Brucella* cells themselves. This was indicated by the lack of detectable agglutinogenic and immunogenic material in the 48-hour whole culture vaccine and their presence in the 13-day whole culture vaccine. Metabolic by-products were further implicated by the culture growth studies and by the cell free vaccines. Removal of the cells completely removed the agglutinin response in guinea pigs, but only partially reduced this response in calves. This indicated that the agglutinogenic material was an index of the amount of metabolic by-products produced, but it did not imply

that agglutinins were an index of protection.

These findings suggested that several approaches may be considered in the preparation of soluble immunogenic agents. One possibility is the artificial lysis of cells in the logarithmic growth phase. The 48-hour culture vaccine as well as the results of Patterson _et al._ (1947) and Sulitzeanu (1958) indicated that the lysis of cultures in the logarithmic growth phase did not produce vaccines with desirable qualities. The vaccine of Patterson and colleagues and the antigens of Sulitzeanu contained agglutinogenic material as well as immunogenic material. Huddleson (1943) successfully removed agglutinogen-free immunogenic material from _Brucella_ cells, but the product was heat labile and sensitive to preservative levels of merthiolate. The second possibility is that natural autolysis which occurs in older cultures may be necessary for the maximal production and liberation of immunogenic material. It is possible that optimum quantities of the material are produced in senescent cells rather than in cells in the logarithmic growth phase.

Another possible factor in the production of a suitable vaccine of this type may have been the inclusion of susceptible host serum in the culture medium to prevent dissociation and to insure that

the organisms were kept in the smooth virulent form throughout the growth period. Thus, a greater quantity of material could have been produced without the danger of obtaining unwanted antigens from dissociated Brucella cells. This use of serum permitted the employment of a liquid rather than a solid culture media, from which soluble metabolic by-products could be more easily extracted. It also made possible the use of older cultures, which have largely been ignored in the past due to the dissociation of the organisms.

Chemical and physical tests indicated that the vaccine was essentially protein in nature. The vaccine had some characteristics in common with various antigens and vaccines extracted from intact Brucella cells by other workers. The fact that it was heat stable and became an irreversible gel following freezing indicated similarities to the material obtained by Paterson et al. (1947) using phenol extraction. Solubility at certain pH ranges indicated that this material was similar to the crude phenol extract of the above investigators i.e., it was apparently readily precipitable along with any acid precipitable material. This vaccine differs from that of Patterson in that immunologically effective dosages did not produce agglutinins in guinea pigs.

This vaccine is similar to that of Huddleson (1943) which produced immunity without an agglutinin response. It differs in that it was not heat or merthiolate sensitive. The vaccine reported here contained two major fractions as determined by paper electrophoretic analysis, while Huddleson's material had three major and one minor component as determined by boundary electrophoresis.

Further studies on the nature of the vaccine, which are not included in the experimental data; indicated that the agglutinogenic material was separated almost completely by 50 per cent ammonium sulfate saturation of the cell-free culture. This material, when subjected to paper electrophoretic analysis, appeared as a single band, which migrated in a manner similar to serum gamma globulin. A further fraction was separated by increasing the concentration of the ammonium sulfate to 71.5 per cent. This fraction contained only minute quantities of agglutinogens. Preliminary evidence indicated that it contained immunogenic properties. Electrophoretic separation of this material was unsatisfactory which may have been due to ammonium sulfate contamination.

Salt fractionation and electrophoretic analysis indicated that the immunogenic material behaved like serum-albumin; while the agglutinogenic material behaved like serum-globulin. It would

appear that salt fractionation and or paper electrophoretic separation of cell-free culture material might prove to be a suitable method in further attempts to purify the immunogenic material. Since the immunogenic material does not seem to be affected by heat, it appears that curtain electrophoretic separation using high voltages might be used successfully.

Although the immunogenic agent is still in a crude state, the results of this study substantiate the original premise that by-products of metabolism may be used to obtain soluble immunogenic agents. The data further indicated that agglutinogens were present in a soluble form and could be successfully separated from immunogens using appropriate methods.

B. Rabbit Studies.

The study of paper electrophoretic patterns of clinically normal rabbits indicated that there was a high incidence of spontaneous pathologic conditions in these animals. Serum protein patterns are an important aid, in addition to routine clinical examination, in selecting rabbits for experimental use. It should be emphasized that the values in this study were obtained under our own laboratory conditions and may not agree exactly with those obtained by others.

In addition, newer methods of staining strips, thinner paper strips, and newer models of the integration scanners tend to give albumin values four to five per cent higher than those observed in this study. However, no matter which method is used, it is felt that no study which includes possible alteration of serum proteins should be undertaken without determining that normal base line values are present at the start of the experimental work.

It is not implied that paper electrophoretic examination of serum is pathognomonic for specific disease conditions. However, it will indicate the major organs where pathological changes may have occurred. This was demonstrated in interstitial nephritis as opposed to amyloid nephrosis. In the former pathological picture the loss of albumin was readily apparent in the serum-protein pattern, but no alteration in the globulins was apparent. In the latter condition the loss of albumin was apparent, also indicating kidney damage, and there was a marked increase in alpha globulins caused by tissue degeneration. These two findings indicated that non-specific necrosis and kidney damage were present. The final diagnosis, of amyloid nephrosis was made by histopathological examination. Paper electrophoretic patterns of various dysproteinemias of humans are considered pathognom-

onic e.g., multiple myeloma, nephrosis and hypogammaglobulinemia (Jenks et al., 1956). Since different species of animals do not show the same serum-protein pattern under similar pathological conditions, (Vesselinovitch, 1959) caution must be used in the interpretation of findings.

Results in the literature suggest that both age and sex may play roles in an immunologic response. The reports are varied and results depend mainly on the agent being studied. In man, males were found to produce higher serum titers against diphtheria (Manson et al., 1954), Streptolysin (Canitano and Morsica, 1955) and Western equine encephalitis (Laveck et al., 1955) than females. Males also showed a higher incidence of histoplasmin sensitivity (Beadenkopfer et al., 1955) and a greater immunity to scarlet fever (Schaefer, 1952) than females. On the other hand, a study in France by Chassagne and Gaignoux (1948) showed a greater incidence of brucellosis in males than females. Young human males were found to be more susceptible to respiratory infections than young females; while the situation was reversed in the adult population (Badger et al., 1953). Ansel and Gauthier (1955) found female mice to be more resistant to candida infection than males. Saslaw et al., (1955) demonstrated that both sexes

of mice show an increased resistance to histoplasmosis with age, but that the female develops this resistance at an earlier age. Male rabbits were shown by Magnuson et al. (1951) to be more susceptible to syphilis than females. Desexing either sex prolonged the incubation period and increased resistance to infection. However, testosterone increased susceptibility in the male; while the incubation period was prolonged in ovario-ectomized rabbits given testosterone.

The effect of sex on the agglutinin titer decline in rabbits was evident in the work reported here. However the role of the steroid hormones was not clear. There appeared to be a reversal in the response in older animals but hormones treatment was apparently stopped too early to fully determine its effect. Further studies are necessary to elucidate the role of steroid hormones in the agglutinin response in rabbits. This may be accomplished by adrenalectomy in addition to castration and ovario-hysterectomy. If any role of the steroid hormones could be determined in an immunologic response it might shed further light on the mechanisms involved in antibody formation.

VII. SUMMARY

A soluble-type vaccine was prepared by the acid-heat extraction of *Brucella abortus* Strain 2308 and its metabolic by-products in Stuart's medium. A comparison was made, in guinea pigs, of 2-day-old and 13-day-old cultures for the preparation of the immunogenic agent. Further comparisons were made in guinea pigs and calves of the 13-day-old culture and cell-free 13-day-old culture vaccine. The agent made from the 2-day-old culture produced no significant protection against various challenge levels of virulent *Br. abortus* Strain 2308. The agent prepared from 13-day-old cultures not only produced significant protection against homologous strain challenge but, produced insignificant serum-agglutination titers at effective dosage levels. The 13-day-old whole and cell-free culture vaccines gave similar results in guinea pigs. In calves the 13-day-old whole-culture vaccine produced higher transient serum-agglutination titers than the 13-day-old cell-free culture vaccine. Protective studies in calves were inconclusive due to inadequate infection in the control animals.

The vaccine was shown to be essentially protein in nature

and contained two distinct fractions on paper electrophoretic examination. The less mobile fraction apparently contained the agglutinogenic material and the more mobile fraction apparently contained the immunogenic material. Removal of the Brucella cells, prior to acid-heat extraction, decreased the less mobile fraction by more than one-half. The degree of serum agglutinin titer response was apparently contingent on this fraction, which indicated the agglutinogens were an index of metabolic by-products, but this did not imply that they were an index of protection.

Paper electrophoretic serum-protein patterns of 15 male and 15 female rabbits were studied. It was determined that the serum fraction percentages of normal rabbits showed little variation, with no differences between sex or breed. The various pathologic conditions were indicated first by serum-protein patterns, and later diagnosed by histopathological examination of necropsy material. An additional 14 rabbits with a natural Eimeria stiedae infection were also investigated. The use of paper electrophoresis as an aid in the selection of normal animals for experimental investigation was demonstrated.

The 13-day-old whole culture vaccine was employed in rabbits

to study serum-agglutinin titer response. The results indicated that the maximum gamma globulin response followed the peak agglutinin response by seven to twenty-one days, this indicated a secondary response the nature of which was not determined. Female rabbits responded to the vaccine with higher initial titers than males and the titer decline was one and one-half times more rapid in the males than in the females. Sex was shown to be the most significant factor in this finding. Castration and ovario-hysterectomy indicated that the above results could be reversed when an estrogenic hormone was given to the castrates and when testosterone was given to the ovario-hysterectomized animals. Insufficient data was available to elucidate the role of steroid hormones in the serum-agglutinin titer response.

VIII. ACKNOWLEDGEMENTS

The author wishes to express his gratitude to the various people who have helped in the various stages of this study. He wishes to express his sincere thanks to Doctor Warren G. Hoag, who brought the author to this school and under whose guidance this work was initiated. He especially wishes to express his gratitude to Doctor John McNeill Sieburth for taking over the difficult task as advisor in the middle of this problem, and for his valuable aid and advice which led to the successful completion of this work. He wishes to thank Doctor Burnham Gross for his aid and counsel as co-advisor during Doctor Sieburth's absence. Gratitude is expressed to Doctor James R. Rooney, II for his histopathological diagnoses and for his advice on the physio-pathologic aspects of this work. The author would like to thank Doctor Douglas F. Watson for his help in surgical procedures, raising of experimental animals, parasitological examinations and his willing aid whenever needed. The author also wishes to express his gratitude to his wife Carol, without whose aid and encouragement this work might not have been accomplished. Thanks are due to

Miss Betty Williams for her typing of the final thesis. Finally, the author wishes to thank the administrative staff of the Virginia Agricultural Experiment Station whose approval for financial support of this project made this study possible.

IX. BIBLIOGRAPHY

Ansel, M. and Gauthier, C. 1955 Experimental candidiasis infection in the mouse by means of intraperitoneal injection with mucin. Influence of sex. "Abstract" Ann. parasitol. Humaine et comp., 30, 312-317. (Biol. Absts., 30, (8202), 1956).

Badger, G. F., Dingle, J. H., Feller, A. E., Hodges, R.G., Jordan, W. S. Jr., and Rammelkamp, C. H. Jr. 1953 A study of illness in a group of Cleveland families II. Incidence of common respiratory diseases. Am. J. Hyg., 58, 31-40.

Bang, B. 1897 Die Aetiologie des seuchenhafen ("infectiosen") Verwerfens. Zeitschr. f. Thiermed. (Jena), 1, 241-278. (Cited by Evans, 1950).

Bang, B. 1906 Infectious abortion in cattle. J. Comp. Pathol., 19, 191-202. (Cited by Plastridge, 1954).

Barner, R. D., Oberst, F. H., and Atkeson, F. W. 1952 Anamnestic reaction as an aid in the differentiation of vaccinal from infection titers of brucellosis (abortus) in cows. Vet. Med., 47, 323-326.

Beadenkopf, W. G., Grayston, J. T., Leonard, J. S., Ward, Jeanne. M., Loosli, G. G., and Hall, C. 1953 Histoplasmin and tuberculin sensitivity among Illinois residents. Am. J. Hyg., 57, 328-344.

Belton, F. C. and Strange, R. E. 1954 Studies on a protective antigen produced in vitro from Bacillus anthracis: Medium and methods of production. Brit. J. Exptl. Pathol., 35, 144-152.

Bendixen, H. C. 1944 A case of infection with Brucella abortus in ampulla ductes deferentis in a bull. Maanedsskr. Dyrl., 56, 1-11. (Cited by Manthei, 1950).

Bendixen, H. C. and Blom, E. 1947 Investigation of brucellosis in the bovine male with special regard to spread of the disease by artificial insemination. Vet. J., 103, 337-345.

Birch, R. R., Gilman, H. E., and Stone, W. S. 1945 The immunity created by vaccination of calves with Brucella abortus Strain 19. Cornell Vet., 35, 110-122.

Blake, G. E. and Manthei, C. A. 1952 The efficiency of the blood, whey, and whole milk tests in ascertaining the brucellosis status of cattle. Proc. Am. Vet. Med. Assoc., 89, 98-104.

Boguth, W. 1953 Papierelektrophoretische Serum untersuchungen bei Haussäugetieren (I. Mitteilung). Zentralbl. fur Veterinarmed., 1, 168-187.

Boguth, W. 1954 Papierelektrophoetische Serum untersuchungen bei Haussäugetieren (II. Mitteilung). Zentralbl. für Veterinarmed., 1, 311-328.

Boivin, A., Mesrobeanu, I., and Mesrobeanu, L. 1933 Extraction d'un complex toxique et antigenique a' partir du bacille d'aertycke. Compt. rend. Soc. Biol., 114, 307-310. Cited by Pennell, 1950.

Boor, A. K. and Tresselt, H. B. 1955 Protection of sheep against anthrax by immunization with an antigen prepared in vitro. Am. J. Vet. Res., 16, 425-428.

Borts, I. H., Harris, D. M., Joynt, M. T., Jennings, J. R., and Jordan, C. F. 1943 A milk-borne epidemic of brucellosis caused by the porcine type of Brucella (Br. suis) in a raw milk supply. J. Am. Med. Assoc., 121, 319-322.

Braude, A. I. and Anderson, Dorothy 1950 The pathogenesis and pathology of experimental brucellosis. In Brucellosis. pp. 50-61. Am. Assoc. Advance Sci., Washington, D.C.

Braun, W. 1946 Dissociation in Brucella abortus: A demonstration of inherent and environmental factors in bacterial

variation. J. Bacteriol., 51, 327-349.

Braun, W. 1950 Variation in the genus Brucella. In Brucellosis. pp. 26-36. Am. Assoc. Advance Sci., Washington, D.C.

Breed, R. S., Murray, E. G. D., and Hitchens, A. P. 1957 Bergey's Manual of Determinative Bacteriology. 7th ed. pp. 404-406. Williams and Wilkins Co., Baltimore.

Bruce, D. 1887 Note on the discovery of a micro-organism in Malta fever. Practitioner (London), 39, 161-170. (Cited by Evans, 1950).

Bryan, H. S., Mansfield, B. S., and Graham, R. 1953 Studies on bovine brucellosis immunization with B.A.I. strain 19 and Huddleson's mucoid vaccines. Proc. 57th Annual Meeting, U.S. Livestock Sanitary Assoc., 124-134.

Buck, J. M. 1930 Studies of vaccination during calfhood to prevent bovine infectious abortion. J. Agric. Res., 41, 667-689.

Buck, J. M. and Cotton, W. E. 1938 Vaccination of calves and yearlings against Bang's disease. U.S.D.A. Tech. Bull. 658, 1-6.

Buddle, M. B. and Boyes, Betty W. 1953 A Brucella mutant causing genital disease in sheep in New Zealand. Aust. Vet. J., 29, 145-153.

Cameron, H. S. and Kendrick, J. W. 1955 The diagnosis of latent bovine brucellosis. J. Am. Vet. Med. Assoc., 126, 131-133.

Cameron, H. S. 1957 A comparison of blood and whey brucellosis tests on 20,000 cows. J. Am. Vet. Med. Assoc., 130, 120-134.

Canitano, P. and Morsica, R. 1955 Antistreptolytic titers amoung healthy individuals in relation to age and sex. "Abstract". Terapia, 40, 181-185. (Biol. Absts. 30, (35001), 1956).

Chassagne, P. and Gaignoux, V. 1948 Enquete sue les brucellosis. Bull. Inst. Nation. Hyg. (Paris), 3, 433-444.

Chiaroni, J. and Nardi, E. 1956 The blood protein picture in the normal rabbit. Atenio purmege, 67, 607-621.

Chopard, P. 1954 Bestimmung der Eiweissfractionen des blut Serums bei den Haustieren mit der Papierelektrophorese, unter Berücksichtigung Verschiedener Faktoren. Schweiz. Arch. für Tierheilk., 96, 252-260.

Coolidge, L. H. 1916 Agglutination test as a means of studying the presence of Bacterium abortus in milk. J. Agric. Res., 5, 871-875.

Cotton, W. E. 1913 The persistence of the bacillus of infectious abortion in the tissues of animals. Am. Vet. Review, 44, 307-318. (Cited by Plastridge, 1954).

Cotton, W. E. and Buck, J. M. 1933 Studies of the skin as a portal of entry for Brucella abortus in pregnant cattle. J. Am. Vet. Med. Assoc., 83, 91-100.

Demoss, R. D. and Bard, R. G. 1957 Physiological and Biochemical techniques. In Microbiological Methods. pp. 169-198. McGraw-Hill Co., Inc., New York.

Dick, J. R., Venzke, W. G., and York, C. A. 1947 A method for differentiating between vaccinal titers and infection titers of Brucella abortus in cattle. J. Am. Vet. Med. Assoc., 111, 255-258.

Edington, B. H. and King, N. B. 1952 A summary of results obtained in Ohio in comparative tests of Huddleson'r Brucella mucoid and strain 19 vaccines. 56th Annual Meeting, Proc. U. S. Livestock Sanitary Assoc., 126-130.

Elberg, S. 1948 The Brucella. In Bacterial and Mycotic Infections of Man. pp. 447-457. Edited by Dubos, R. J., Lippincott Company, Philadelphia.

Evans, Alice, C. 1947 Brucellosis in the United States. Am. J. Pub. Health, 37, 139-151.

Evans, Alice C. 1950 Comments on the early history of human brucellosis. In Brucellosis. pp. 1-8. Am. Assoc. Advance Sci., Washington, D. C.

Fisher, R. A. and Yates, F. 1949 Statistical Tables. 3rd ed. 112 p. Hafner Publishing Co. Inc., New York.

Fitch, C. P., Donham, C. R., Bishop, Lucille M., and Boyd, W. L. 1930 Studies on the tube agglutination test for the diagnosis of Bang's disease (contagious abortion). Univ. Minn. Agric. Exper. Stat. Bull. Tech. Bull., 73, 1-56.

Fleischhauer, G. 1937 Die Abortus-Bang-Ringsprobe (ABR) zur Festellung von bangverdächtigen voll milchproben. Berlin Tierärztl. Wochschr., 53, 527-528. (Cited by Roepke, 1950).

Fleischhauer, G. and Hermann, G. 1938 Ueber weitere Erfarhrungen mit der Abortus-Bang-Ringprobe (ABR) bei der Untersuchung von Milchproben auf Abortus Bang. Berlin Tierärztl. Wochschr., 54, 333-334. (Cited by Roepke, 1950).

Freund, J., Thomson, K. J., Hough, H. B., Sommer, H. E., and Pisani, T. M. 1948 Antibody formation and sensitization with the aid of adjuvants. J. Immunol., 60, 383-398.

Gerhardt, P. 1958 The nutrition of brucellae. Bact. Reviews, 22, 81-98.

Giltner, W. and Bandeen, S. G. 1920 The bacteriology of the reproductive organs of the cow and its relation to that of the meconium of the calf. J. Am. Vet. Med. Assoc., 57, 46-57.

Gladstone, G. P. 1946 Immunity to anthrax: Protective antigen present in cell-free culture filtrates. Brit. J. Exptl. Pathol., 27, 394-410.

Grinsted, F. 1909 The agglutination test for the diagnosis of infectious abortion in cattle. Maanedsskr. Dyrl., 21, 395. (Cited by Huddleson, 1943b).

Gwatkin, R. 1932 Incidence of Brucella abortus in the fetal membranes of full-time, reacting cows. Cornell Vet., 22, 62-66.

Gwatkin, R. and Panisset, M. 1933 The campaign against epizootic abortion in Ontario. The role of the Ontario Research Foundation. Bull. de l'Acad. Vet., 399-407. (Cited by Plastridge, 1954).

Haring, C. W. and Traum, J. 1943 The effect of Brucella abortus Strain 19 on cattle of various ages and its bearing on adult cattle vaccination. Proc. 47th Annual Meeting, U. S. Livestock Sanitary Assoc., 42-46.

Hart, G. H. and Traum, J. 1925 The relation of the subcutaneous administration of living Bacterium abortum to the immunity and carrier problem of bovine infectious abortion. Univ. of Calif. Agric. Exper, Stat. Tech. Paper, No. 19, 1-50.

Hasseltine, H. E. 1930 The relationship of human and animal brucelliasis. J. Am. Vet. Med. Assoc., 29, 330-338.

Hawk, P. B., Oser, B. L., and Summerson, W. H. 1947 Practical Physiological Chemistry. 12th ed. 1323 p. Blakiston Co., Philadelphia.

Henry, B. S. 1933 Dissociation in the genus Brucella. J. Infectious Diseases, 52, 374-402.

Hess, W. R. and Roepke, M. H. 1951 A non-specific Brucella-agglutinating substance in bovine serum. Proc. Soc. Exptl. Biol. and Med., 77, 469-472.

Hess, W. R. 1953 Studies on a non-specific Brucella-agglutinating substance in bovine serum I. The differentiation of the specific and non-specific agglutinins by heat

treatment. Am. J. Vet. Res., 14, 192-194.

Hoag, W. G. 1955 Unpublished data. Virginia Agric. Exper. Stat., Virginia Polytechnic Institute.

Hoag, W. G. and Bell, W. B. 1955 An immunogenic agent for the protection of cattle against Leptospira pomona. Am. J. Vet. Res., 16, 381-385.

Hoag, W. G. and Allen, R. C. 1959 Studies on an immunogenic agent against Brucella abortus. I. Titration of a new soluble immunogenic agent against experimental infections in guinea pigs. Am. J. Vet. Res., 20, 162-165.

Huddleson, I. F. and Carlson, E. R. 1926 A rapid method for performing the agglutination test in the serum diagnosis of Bang's abortion disease. J. Am. Vet. Med. Assoc., 70, 229-233.

Huddleson, I. F. and Abell, F. 1928 Rapid macroscopic agglutination for the serum diagnosis of Bang's abortion disease. J. Infectious Diseases, 42, 242-247.

Huddleson, I. F. 1943a The immunization of guinea pigs against Brucella infection. Mich. State Coll. Agric. Exper. Stat. Tech. Bull., 182, 45-56.

Huddleson, I. F. 1943b Brucellosis in Man and Animals. Rev. Ed. 379 p. Commonwealth Fund, New York.

Huddleson, I. F., Wood, E. E., Cressman, A. R., and Bennett, G. R. 1945 The bactericidal action of bovine blood for Brucella and its possible significance. J. Bacteriol., 50, 261-277.

Huddleson, I. F. 1946 The mucoid phase of the genus Brucella. Am. J. Vet. Res., 7, 5-10.

Huddleson, I. F. 1947 The immunization of guinea pigs with mucoid phases of Brucella. Am. J. Vet. Res., 8, 374-379.

Huddleson, I. F. and Bennett, G. R. 1948 The vaccinal immunizing value of a mucoid-growth phase of Brucella suis against brucellosis in cattle. Mich. Agric. Exper. Stat. Quar. Bull., 31, 139-159.

Huddleson, I. F. 1951 Studies in brucellosis, III Part I. The dissociation genus Brucella. Memoir 6, pp. 7-62. Mich. State Coll. Agric. Exper. Stat. East Lansing, Mich.

Huddleson, I. F. 1957 Factors that promote the growth of Brucella in the presence of blood. Bull. Wld. Hlth. Org., 16, 929-938.

Jencks, W. P., Smith, E. R. B., and Durrum, E. L. 1956 The clinical significance of the analysis of serum protein distribution by filter paper electrophoresis. Am. Jour. Med., 21, 387. (Cited by Vessolinovitch, 1959).

Kerr, W. R., Pearson, J. K. L., and Rankin, J. E. F. 1958 A brucellosis survey in dairy herds with particular reference to diagnostic methods including the vaginal mucus test. Vet. Rec., 70, 503-509.

King, N. B., Venzke, W. G., and Edington, B. H. 1953 The application of a suggested method for differentiating vaccinal and infection titers in cattle known to be infected with Brucella abortus. J. Am. Vet. Med. Assoc., 123, 121-125.

King, R. O. C. 1940 Brucella infection in the bull. A progress report of mating experiments with naturally infected bulls. Austral. Vet. J., 16, 117. (Cited by Huddleson, 1943b).

Kramer, C. Y. 1959 Statistics Department, Virginia Polytechnic Institute. Personal Communication.

Laveck, G. D., Winn, J. F., and Welch, Sarah F. 1955 Inapparent infection with Western equine encephalitis virus: Epidemiologic observations. Am. J. Publ. Health, 45, 25-28.

McCullough, N. B. 1950 Laboratory tests in brucellosis. In Brucellosis. pp. 116-121. Am. Assoc. Advance. Sci. Washington, D. C.

McDiarmid, A. and Sutherland, F. B. 1957 A comparison of the immunity produced in guinea-pigs by the inoculation of living heat-killed and adjuvant vaccines prepared from Br. abortus strains 45/20 and S. 19. Vet. Rec., 69, 1067-1070.

McEwen, A. D. and Samuel, I. McA. 1955 Brucella abortus: Heat stable protective antigen revealed by adjuvant and present in a "rough" variant strain 45/20: Immunization experiments on guinea pigs. Vet. Rec., 67, 546-548.

McFadyean, J. and Stockman, S. 1916 Epizootic abortion in cattle. Rpt. Dept. Come Bd. Agric. Fish. Gt. Brit., Part 1, 1-43. (Cited by Traum, J. 1950).

MacNeal, W. J. and Kerr, J. 1910 Bacillus abortus of Bang, the cause of contagious abortion in cattle. J. Infectious diseases, 7, 469-475.

Magnuson, H. J., Rosenau, Barbara J., and Greenberg, B. G. 1951 The effects of sex castration and testosterone upon the susceptibility of rabbits to experimental syphilis. Amer. J. Syphillis, Gonnorrhea and Venereal Dis., 35, 146-163.

Maitland, H. B. and Guérault, A. 1958 Some surface components of Haemophilus pertussis: Immunizing antigen, histamine-sensitizing factor and agglutinogen. J. Pathol. and Bacteriol., 76, 257-274.

Manthei, C. A. 1950 Brucellosis in cattle. In Brucellosis pp. 172-187. Am. Assoc. Advance. of Sci., Washington, D. C.

Manthei, C. A. 1953 Review of suggested methods for differentiation of vaccinal and infection serum agglutinin titers for brucellosis in cattle. Proc. 57th Annual Meeting, U. S. Livestock Sanitary Assoc., 135-143.

Marsh, H. and Wilkins, H. F. 1948 Control of brucellosis in range cattle. Proc. 52nd Annual Meeting, U. S. Livestock Sanitary Assoc., 184-186.

Mason, J. H., Robinson, Mary, Preiss, Minnie and Christensen, P. A. 1954 Immunization against diphtheria: Comparison of A.D.F. P.T.A.P. and A.P.T. in man. "Abstract" S. African Med. Jour. 28, 1071-1074. (Biol. Absts. 29, (26445)).

Miles, A. A. 1939a The antigenic surface of smooth Brucella abortus and meletensis. Brit. J. Exptl. Pathol., 20, 63-82.

Miles, A. A. and Pirie, N. W. 1939b The properties of antigenic preparations from Brucella melitensis: I. Chemical and Physical properties of bacterial fractions. Brit. J. Exptl. Pathol., 20, 83-98.

Miles, A. A. and Pirie, N. W. 1939c The properties of antigenic preparations from Brucella melitensis: II. Serological properties of the antigens. Brit. J. Exptl. Pathol., 20, 109-121.

Miles, A. A. and Pirie, N. W. 1939d The properties of antigenic preparations from Brucella melitensis: III. The biological properties of the antigen and the products of gentle hydrolysis. Brit. J. Exptl. Pathol., 20, 278-296.

Minett, F. C. and Martin, W. J. 1936 Influence of mastitis and of Brucella abortus infection upon the milk yield of cows. J. Dairy Res., 7, 122-144.

Mingle, C. K. 1946 Bovine brucellosis research. Proc. 50th Annual Meeting, U. S. Livestock Sanitary Assoc., 265-273.

Olitzki, A. L. and Sulitzeanu, D. 1957 The antigenic structure in the genus Brucella. pp. 321-326. Proc. of the Third international Meeting of Biological Standardization, Opatija, Sept. 2-6.

Olitzki, A. L. and Sulitzeanu, D. 1958 Studies on the antigenic structure of Brucella suis with the aid of the agar gel precipitation technique. I. The resistance of antigens to physical, chemical and enzymatic treatment. Brit. J. Exptl. Pathol., 39, 219-227.

Olitzki, A. L. and Gurevitch, J. 1933 Zentralbl. Bakt. I. Orig., 128, 112. (Cited by Olitzki and Sulitzeanu, 1958).

Ouchterlony, O. 1948 In vitro method for testing the toxin producing capacity of diphtheria bacteria. Acta. Path. et microbiol. Scavid., 25, 186-191. "Abstract" (Biol. Absts. 23, (4372) 1949).

Paterson, J. S., Pirie, N. W. and Stableforth, A. W. 1947 Protective antigens isolated from Br. abortus. Brit. J. Exptl. Pathol., 28, 223-236.

Pennell, R. B. and Huddleson, I. F. 1937 Chemical Constitution and biological properties of the endo-antigen of the Brucella group of organisms, and their interaction with Brucella specific sera. Mich. State Coll. Agric. Exper. Sta. Tech. Bull., 156. 1-31.

Pennell, R. B. 1950 The chemistry of Brucella organisms. In Brucellosis. pp. 37-49. Am. Assoc. Advanc. Sci., Washington, D. C.

Plastridge, W. N. 1954 Bovine brucellosis. Proc. 58th Annual Meeting, U. S. Livestock Sanitary Assoc. 135-179.

Puziss, M. and Wright, G. G. 1954 Studies on immunity in Anthrax. IV. Factors influencing elaboration of the protective antigen of Bacillus anthracis in chemically defined media. J. Bacteriol., 68, 474-482.

Reed, L. J. and Muench, H. 1938 A simple method of estimating fifty per cent endpoints. Am. J. Hyg., 27, 493-497.

Renoux, G. and Mahaffry, L. 1955 Wld. Hlth. Org. FAO/WHO

joint advisory panel on Brucellosis Feb. 10, 1955. (Cited by Olitzki and Sulitzeanu, 1958).

Roepke, M. H. 1950 The Brucella abortus ring test. In Brucellosis. pp. 126-135. Am. Assoc. Advance Sci., Washington, D. C.

Roepke, M. H., Paterson, K. G., Driver, F. C., Clausen, L. B., Olson, L., and Wentworth, J. E. 1950 The Brucella abortus ring test. Am. J. Vet. Res., 11, 199-205.

Rooney, J. R. II. 1957 Paper electrophoresis of bovine serum. Am. J. Vet. Res., 18, 67-72.

Rosenbasch, C. 1947 Prevention de la brucellosis por medio de una vacuna base de cepas atenuadas no aglutinogenas (cepas MBB). Mem. Primer Cong. Macional de la Brucellosis del Uraguay (Dec. 1947), 218-237.

Saslaw, S. and Schaefer, J. 1955 Relation of sex and age to resistance of mice to experimental histoplasma infections. Proc. Soc. Exptl. Biol. and Med., 90, 400-402.

Schaefer, W. 1952 Age range and disposition of scarlatina: Sex distribution of scarlatina. "Abstract" Zentralbl Bakt Abt 1. Orig 159 (112) 48-55. (Biol. Abst. 28, (6487), 1954).

Schlingman, A. A. and Manning, M. S., 1952 Further observations on inactivated Brucella abortus as an immunizing agent in cattle. Nort. Amj. Vet., 33, 397-405.

Schroeder, E. C. and Cotton, W. E. 1911 The bacillus of infectious abortion found in milk. 28th Annual Rpt., B.A.I., U.S.D.A., 139-146.

Schroeder, E. C. 1922 Bureau of Animal Industry Investigation on bovine infectious abortion. J. Am. Vet. Med. Assoc., 522. (Cited by Huddleson, 1943b).

Seit, B. 1944 Kastningssmitte Ved Kunstis Imseminering.

Maunedsskr. Dyrl., 56 12. (Cited by Manthei, 1950).

Siero, F. 1954 Studies on comparative brucella immunity with agglutinogenic and non-agglutinogenic vaccines. Am. J. Vet. Res., 15, 417-424.

Smith, T. and Fabyan, M. 1912 Über die pathogen uvirkung des Bacillus abortus bang. Zentralbl. f. Bakt. 1. Abt., Orig., 61, 549-555. (Cited by Elberg, 1948).

Snedecor, G. W. 1946 Statistical Methods. 4th ed. pp. 214-317. Iowa State College Press, Ames, Iowa.

Soule, M. H. 1950 A summary of the present knowledge of brucellosis. In Brucellosis. pp. 247-255. Am. Assoc. Advance. Sci., Washington, D. C.

Spector, W. S. 1956 ed. Handbook of Biological Data. pp. 25. W. B. Saunders Co., Philadelphia, Penn.

Spink, W. W. 1956 The Nature of Brucellosis. 464 p. University of Minnesota Press, Minneapolis, Minn.

Spink, W. W. 1957 The significance of bacterial hypersensitivity in human brucellosis: Studies on infection due to strain 19 Brucella abortus. Ann. Internal. Med., 47, 861-874.

Spinco 1955 Tech. Bull. 6072A.

Stockman, S. 1914 Epizootic abortion. J. Comp. Path. Therap., 27, 237-246. (Cited by Traum, 1950).

Stoenner, H. G. and Lackman, D. B. 1957 A new species of Brucella isolated from the desert wood rat, Neotoma lepida Thomas. Am. J. Vet. Res., 18, 947-951.

Stuart, R. D. 1946 Preparation and use of a simple culture medium for Leptospirae. J. Pathol. and Bact., 58, 343-349.

Sulitzeanu, D. 1958 Relationship of agar diffusion patterns to agglutinating and protective properties of Brucella suis

antisera. Brit. J. Exper. Pathol., 39, 367-375.

Thorebecke, G. J. and Keuning, F. S. 1956 Antibody and gamma globulin formation in vitro in hemopoietic organs. J. Infectious Diseases, 98, 157-171.

Thorne, C. B. and Belton, F. C. 1957 An agar-diffusion method for titrating Bacillus anthracis immunizing antigen and its application to a study of antigen production. J. Gen. Microbiol., 17, 505-516.

Traum, J. 1914 Infectious abortion investigations in pigs. Ann. Rept. Dept. Agric., Washington, D. C., p. 86.

Traum, J. and Maderious, W. E. 1947 The interpretation of whey agglutination test results in cows vaccinated with Brucella abortus strain 19. Am. J. Vet. Res., 8, 244-246.

Traum, J. 1950 The control of brucellosis in animals by the use of vaccine. In Brucellosis pp. 225-235. Am. Assoc. Advance Sci., Washington, D. C.

Van den Ende, M., Polson, A., and Turner, G. S. 1957 Experiments with the soluble antigen of rabies in suckling mice. J. Hyg., 55, 361-373.

Vanghelovici, M., Damboviceanu, A., Barber, C. and Pop, A. 1938 Complete antigens of the Brucella. Microchemical analysis. Compt. rend. Soc. Biol., 127, 738. (Cited by Pennell, 1950).

Vesselinovitch, S. D. 1959 The analysis of serum proteins of domestic animals by filter-paper electrophoresis. A review. Cornell Vet., 49, 82-96.

White, G. C., Johnson, R. E., Rettger, L. F., and McAlphine, J. G. 1925 Infectious abortion in cattle. Eighth report. Some economic phases of Bacterium abortus infection and other observations in dairy herds. Storrs (Conn.) Agric. Exper. Station Bull., 135, 407-428.

White, G. C., Johnson, R. E., Rettger, L. F., and McAlpine, J. G. 1929 Bovine infectious abortion- Eleventh Report- Increased productivity of an abortion-free dairy herd. Storrs (Conn.) Agric. Exper. Station Bull. 154, 133-147.

Wilson, G. S. and Miles, A. A. 1932 The serological differentiation of smooth strains of the Brucella group. Brit. J. Exptl. Pathol., 13, 1-12.

Wright, A. E. and Semple, D. 1897 On the employment of dead bacteria in the serum diagnosis of typhoid and Malta fever. Brit. Med. Jour., 1, 1214. (Cited by Huddleson, 1943 b).

Wright, G. G., Hedberg, M. A., and Slein, J. B. 1954 Studies on immunity in anthrax. III. Elaboration of protective antigen in a chemically-defined, non-protein medium. J. Immunol., 72, 263-269.

Zeller, M. and Stockmeyer, W. 1936 Trials of some anti-abortus vaccines in cattle. Vet. Bul., 6, pp. 643.

Zobell, C. F. and Meyer, K. F. 1932 Metabolism studies in the Brucella group. IX. Physicochemical requirements in synthetic mediums. J. Infectious Diseases, 51, 361-381.

The two page vita has been removed from the scanned document.

Page 1 of 2

The two page vita has been removed from the scanned document.

Page 2 of 2

XI. APPENDICES

A. Bacteriological Culture Media

1. Brucella Broth (Albimi)

	gm/liter
Pepture "M"	20.0
Dextrose CR	1.0
Yeast Autolysate	2.0
Sodium Chloride	5.0
Sodium Bisulfite	0.1

2. Huddleson's Host Blood Medium

To a 50-ml serum bottle is added 0.5 gm of Duolite C-3 $^{H+}$ ion exchange resin (Chemical Process Co., Redwood City, California) and two ml of five per cent sodium citrate. The bottle is stoppered with cotton and autoclaved at 120° C for 20 minutes (bottles prepared in this manner may be closed with a rubber stopper and held indefinitely). Blood is collected asceptically, either with or without sodium citrate, and 10 ml are added to the bottle. The blood and resin are mixed well and the bottles are closed immediately with a sterile rubber stopper to retain the CO_2 resulting from the decomposition of $NaHCO_2$ by the H^+ ions, and are incubated at 37°C.

Preparation of Duolite C-3 $^{H+}$: Granules are washed, dried

and stored a closed bottle. The resin granules of each lot are titrateolood in order to determine the amount that should be used totain a pH of 6.0 - 6.2.

3. Stuart's Medium

	Per/liter
Asparagine	0.132 gm
NH_4 Cl	0.268 gm
$MgCl_2$	0.191 gm
NaCl	1.925 gm
Glycerol	5.0 ml
Sorensen Buffer pH 7.6	81.1 ml
H_2O	to make 1 liter

Steor boil 30 minutes, cool and filter through Whatman #3 pap remove precipitated phosphates. Autoclave 30 minuteool and add 10 per cent sterile rabbit serum.

4. Trypticase Soy Broth (BBL)

	gm/liter
Trypticase	17.0
Phytone	3.0
Sodium Chloride	5.0
Dipotassium phosphate	2.5
Dextrose	2.5
Final pH 7.3 ±	

B. Animal Feed Formulas

1. Pelleted Rabbit Feed

Guaranteed Analysis:

Crude Protein	(minimum) ...	18.00%
Crude Fat	(minimum) ...	2.50%
Crude Fiber	(maximum)...	10.00%
Nitrogen Free Extract ..	(minimum) ...	47.00%

Ingredients:

Pounds:	
400	Wheat Flour Middlings
721.25	Yellow Corn Meal
200	Ground Oats
250	Alfalfa Meal
275	Dehulled Soybean Oil Meal
100	Linseed Oil Meal
20	(1%) Ground Limestone
20	(1%) Defluorinated Phosphate
10	(.5%) Iodized Salt
1/2	(.025%) Manganese Sulfate
3	Antibiotic Feed Supplement, Vitamin B_{12} Supplement, D-Activated Plant Sterol

2. Calf Developer

Guaranteed analysis:

Crude Protein	(min.).......	18.00%
Crude Fat	(min.).......	4.00%
Crude Fiber	(max.)	8.00%
Nitrogen Free Extract.......	(min.).......	45.00%

Open formula:

Pounds:	
300	Wheat Bran
157 3/4	Coarse Cracked Corn
240	Corn Meal
300	Crimped Oats
150	Corn Distillers' Dried Grains
13	Antibiotic Feed Supplement
100	Alfalfa Meal
225	Soybean Oil Meal
200	Linseed Oil Meal
100	Dried Whole Whey
20	Animal Fat (preserved with butylated hydroxyanisole, propyl gallate, citric acid and propylene glycol)
150	Cane Molasses
9	Vitamin A Feeding Oil
13	(.65%) Ground Limestone
8	(.4%) Defluorinated Phosphate
10	(.5%) Iodized Salt
	D-Activated Plant Sterol

3. Clovite

Clovite is a concentrated, vitamin feed-supplement containing both oil- and water-soluble vitamins in a golden-yellow fish-oil and vegetable oilmeal base.

Formula, with guaranteed minimum vitamin content:

Vitamin A	-	Per gram, 242.5 U.S.P. Units Per pound, 110,000 U.S.P. Units
Vitamin D_3	-	Per gram, 110.2 I.C. Units Per pound, 50,000 I.C. Units
Vitamin D_2	-	Per gram, 440.9 U.S.P. Units Per pound, 200,000 U.S.P. Units
Vitamin B_{12}	-	Per gram, 1.101 mcg. Per pound, 500 mcg.

Thiamine, riboflavin, pantothenic acid, niacin and choline, in supplemental amounts.

Dicalcium phosphate, 2%

Suitable base, Q.S.

ABSTRACT

ALLEN, R. C. (Virginia Polytechnic Institute, Blacksburg) A soluble acid-heat extracted Brucella vaccine: Immunological and physiological studies in guinea pigs, rabbits and calves. 143 p. Dissertation. 1959.-- A soluble-type vaccine was prepared by the acid-heat extraction of *Brucella abortus* Strain 2308 and its metabolic by-products in Stuart's medium. A comparison was made, in guinea pigs, of 2-day-old and 13-day-old cultures for the preparation of the immunogenic agent. Further comparisons were made in guinea pigs and calves of the 13-day-old culture and cell-free 13-day-old culture vaccine. The agent made from the 2-day-old culture produced no significant protection against various challenge levels of virulent *Br. abortus* Strain 2308. The agent prepared from 13-day-old cultures not only produced significant protection against homologous strain challenge but, produced insignificant serum-agglutination titers at effective dosage levels. The 13-day-old whole and cell-free culture vaccines gave similar results in guinea pigs. In calves the 13-day-old whole-culture vaccine produced higher transient serum-agglutination titers than the 13-day-old cell-free culture vaccine.

Protective studies in calves were inconclusive due to inadequate infection in the control animals.

The vaccine was shown to be essentially protein in nature and contained two distinct fractions on paper electrophoretic examination. The less mobile fraction apparently contained the agglutinogenic material and the more mobile fraction apparently contained the immunogenic material. Removal of the *Brucella* cells, prior to acid-heat extraction, decreased the less mobile fraction by more than one-half. The degree of serum agglutinin titer response was apparently contingent on this fraction, which indicated the agglutinogens were an index of metabolic by-products, but this did not imply that they were an index of protection.

Paper electrophoretic serum-protein patterns of 15 male and 15 female rabbits were studied. It was determined that the serum fraction percentages of normal rabbits showed little variation, with no differences between sex or breed. The various pathologic conditions were indicated first by serum-protein patterns, and later diagnosed by histopathological examination of necropsy material. An additional 14 rabbits with a natural *Eimeria stiedae* infection were also investigated. The use of paper

electrophoresis as an aid in the selection of normal animals for experimental investigation was demonstrated.

The 13-day-old whole culture vaccine was employed in rabbits to study serum-agglutinin titer response. The results indicated that the maximum gamma globulin response followed the peak agglutinin response by seven to twenty-one days, this indicated a secondary response the nature of which was not determined. Female rabbits responded to the vaccine with higher initial titers than males and the titer decline was one and one-half times more rapid in the males than in the females. Sex was shown to be the most significant factor in this finding. Castration and ovario-hysterectomy indicated that the above results could be reversed when an estrogenic hormone was given to the castrates and when testosterone was given to the ovario-hysterectomized animals. Insufficient data was available to elucidate the role of steroid hormones in the serum-agglutinin titer response.