A STUDY OF THE ANTIBODIES PRODUCED IN CHICKENS IN RESPONSE TO INJECTIONS OF PHENOL EXTRACTED BRUCELLA ABORTUS

by

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Submitted to the Faculty of the Graduate Division
of the Medical College of Georgia in Partial Fulfillment
of the Requirements for the Degree of
Master of Science
September
1966

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This thesis submitted by Albert Nathan Rabin has been examined and approved by an appointed committee of the faculty of the Graduate Division of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis has received final approval with reference to content, form and accuracy of presentation.

This thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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October 19, 1966
Date

ACKNOWLEDGEMENTS

I wish to acknowledge and thank:

Dr. Stanley S. Lefkowitz, my advisor and friend, for sponsoring my research and for his valuable assistance in helping me complete this phase of my graduate studies;

Dr. Catherine E. Roesel for her council in important aspects of my research;

Drs. Edward Gardner, Jr., John Howard, Sam Singal, and Arthur White for generously allowing me to utilize their laboratory facilities;

Dr. J. Warren Banister for his good efforts at the inception of this project.

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INTRODUCTION

A. Statement of the Problem:

In immunological diseases of man such as systemic lupus erythematosis (SLE), the presence of antibodies to nuclear components is well established. Miescher et al (1960) indicated the existence of at least four classes of antibodies associated with this disease. These investigators reported that in SLE, antibodies are formed against: (1) nucleoproteins, (2) soluble extracts of cell nuclei, (3) histones, and (4) deoxyribonucleic acid (DNA). Although anti-DNA antibodies occur spontaneously in SLE, immunization with purified DNA has failed to stimulate antibody production. Detectable antibodies to highly purified DNA have not been demonstrated despite a variety of efforts by a number of other competent investigators. The present concensus among immunologists and immunochemists seems to be that pure DNA is not antigenic.

In 1958, Phillips et al injected rabbits with a phenol extracted bacterial DNA preparation containing twenty per cent protein (Braun et al, 1957) and obtained results that suggest that DNA possesses antigenic specificity. Evidence for specificity was based upon the appearance of a Feulgen positive precipitin band in gel diffusion tests indicating the presence of DNA. Reaction of antiserum with DNA-ase treated antigen prevented the appearance of a Feulgen positive precipitin band.

The production of antibodies to antigens associated with DNA has thus far been studied primarily in rodents. It would be of interest to immunize animals other than rodents with phenol extracts containing DNA and

investigate the nature of the antibody response. Consequently the investigation to be reported in this paper is a study of the antibodies produced by the common chicken (Gallus domesticus) in response to injections of phenol extractions of Brucella abortus.

The chicken was chosen for this work because it falls within a different class of the phylum Chordata than the experimental animals which have been used in previous studies of this type. Campbell et al (1964) indicated that chickens are better producers of precipitating antibodies than are rabbits. In addition, no reports on the use of chickens for this purpose have been encountered in searching the literature.

Brucella abortus was chosen as the source of DNA. Extraction procedures were essentially those of Braun et al (1957). It is the purpose of the present study to characterize phenol extracts of Brucella abortus utilizing both chemical and serological analyses.

B. Review of the Related Literature:

The question of whether or not DNA possesses antigenic specificity has been studied for over a quarter of a century. Sevag et al (1938) isolated nucleic acid by mild hydrolysis of streptococcal nucleoprotein and shaking the resultant solution with chloroform and amyl alcohol. A total of twenty-five milligrams of streptococcal nucleic acid was used to immunize rabbits utilizing a series of five injections over a two week period. Of four nucleic acid antisera samples, only two were serologically active. One nucleic acid preparation reacted with homologous serum in precipitation tests in titers as high as 1:10,000 and in complement fixation tests as high as 1:8000. All reactions with heterologous sera gave negative results. A second nucleic acid preparation gave precipitation titers of 1:100,000 and complement fixation titer of 1:62,000 with homologous serum, while heterologous serum gave precipitation and complement fixation titers of 1:1,000 and 1:2,500 respectively.

Lackman et al (1941) inoculated horses and rabbits with streptococci, pneumococci, and acid precipitated streptococcal nucleoproteins. They studied the serology of nucleic acid obtained from streptococci, pneumococci, bull spermatozoa, yeast cells, tobacco mosaic viruses, tubercle bacilli, and calf thymus by reacting these nucleic acids with the horse and rabbit antisera. The reaction of nucleic acids with antibacterial sera indicated that nucleic acids gave a specific precipitates with equine antipneumococcal sera. Since precipitins of corresponding reactivity are usually not found in normal sera or any other kind of sera, it was concluded that they were formed during immunization with pneumococci. It was not determined whether the precipitins arose as a direct immunity

response to the nucleoprotein antigens of pneumococci.

Avery, et al (1944) in studying pneumococcal transformation, induced transformation by inoculating a DNA fraction isolated from pneumococcus Type III into dogs and rabbits. The serologic results obtained in connection with this study showed irregularity and broad cross reactions in relation to DNA antigenicity. These investigators claimed DNA is not antigenically active.

Blix et al (1954) immunized rabbits with DNA preparations from calf thymus. They reported their studies to indicate that samples of pure, freshly prepared calf thymus DNA invariably gave rise to high titer specific complement fixing antibodies upon injection into rabbits. Nucleic acids from older calf thymus preparations were reported to be also antigenic but the titers obtained were lower. In addition, the following results were reported: The DNA antisera cross reacted with histone and with DNA. Histone was found to be non-antigenic. The DNA did not give rise to anaphylaxis, but a moderate degree of passive anaphylaxis was observed in the guinea pig.

The studies of Blix et al (1954) failed to reveal whether their antibodies were specific for DNA or for some other unrelated antigen present. Also, the results obtained are questionable when either antigen or antibody is anti-complementary as was found to be the case. In addition, complement fixation was detected by mixing dilutions of antigen with constant amounts of antiserum, leaving open to equivocation the reported titers of antibody. In relation to this, Haskova et al (1959) reported that DNA isolated by different methods from fresh or frozen calf thymus did not, under the experimental conditions established, induce antibody formation which could be detected in rabbit serum by complement fixation, ring precipitation,

Ouchterlony gel diffusion, or passive hemagglutination with formalinized erythrocytes.

All attempts to induce antibodies against nucleic acids by injecting heterologous hosts with pure DNA or RNA have been reported as negative. Such failures have been interpreted to mean that although nucleic acids could theoretically be considered antigenic, they are in reality non-antigenic (Plescia et al, 1963). Nucleic acids may actually be antigenic, but according to Panijel (1963) it is difficult to demonstrate the antigenicity, because when injected, DNA is rapidly decomposed by the host's DNAase.

Since highly purified DNA has not been demonstrated to be antigenic, the question arises: Is DNA a hapten, and does DNA need the attached protein? The problem of too much protein means that the antibodies produced would be non-specific for DNA, and too little protein would be insufficient to stimulate antibody formation.

Braun, et al (1957) extracted highly polymerized bacterial DNA from Brucella abortus. The cells were killed with phenol, treated with amyl alcohol and chloroform, and the DNA precipitated with 95 per cent ethanol. The fibrous precipitate was analyzed to contain approximately eighty per cent DNA, twenty per cent protein and less than 1.5 per cent RNA. These investigators reported that although the resulting material most closely resembles DNA and is referred to as such, and despite the fact that the material is biologically independent (as demonstrated in transformation of bacteria) of the attached protein the resultant isolates may actually be an unusually stable nucleoprotein.

The Brucella DNA extract was reported to have a phosphorus extinction

coefficient of 8,900 at 260 mu and a nitrogen-phosphorus mole ratio at various stages of extraction to be 2.94, 2.29, 2.39, 3.33 and 5.00 (Braun et al 1957). Chargaff and Davidson (1955) determined that purified DNA has a phosphorus coefficient of 6,600 at 260 mu and a nitrogen-phosphorus mole ratio of 3.60.

Braun et al (1957) did not report any inorganic salt contamination which may have occurred during the extraction procedures nor did they report any attempt to remove extraneous material which may have precipitated with the DNA. These workers did not determine the DNA content on a dry weight basis. After precipitation, their DNA extract was suspended in normal saline for testing and/or storage. The amount of DNA used for injection was determined by the Dische-Stumpf reaction which measures the deoxypentose content of the dissolved sample.

Phillips et al (1958) immunized rabbits with phenol extracted Brucella abortus DNA. The antiserum was tested for precipitating antibodies against Brucella DNA by the Ouchterlony gel diffusion technic. Five clearly distinguishable bands of precipitins developed in the gel. Absorption of the antisera with formalin killed whole cells or with insoluble cellular residue remaining after phenol extraction failed to alter the precipitin pattern. One of the precipitation bands gave a positive Feulgen stain indicating the presence of DNA. After treatment of the test antigen with DNA-ase, a noticeable decrease in the intensity of the Feulgen positive band took place. DNA-ase alone did not react with the antiserum.

The appearance of a Feulgen positive precipitation band in the gel diffusion and the diminished intensity of this band following treatment with DNA-ase was the method by which Phillips et al (1958) demonstrated antibodies to Brucella DNA extract in rabbit serum. They further reported that DNA

could have precipitated without being antigenic because it may have been bound to other antigens or that it might have coprecipitated with an unrelated antigen-antibody precipitate.

Miescher et al (1960) immunized rabbits and guinea pigs with thymus and liver nuclei, histone, calf thymus nucleoprotein, calf thymus DNA, salmon sperm DNA and Brucella abortus DNA. All attempts at detecting antinuclear precipitins in the guinea pig failed. It appears that nucleoproteins act as a weak antigen in rabbits giving rise to antibody production in about half the immunized animals. When cell nuclei are used as antigens, the results depend upon the intensity of immunization. The antiserum was shown to cross react with DNA from various sources, such as was observed between calf thymus and Brucella abortus DNA.

Braun et al (1959) report that DNA-ase sensitivity has not been observed with every batch of the phenol extracted antigen. Some batches contain components that lose antigenic reactivity following DNA-ase exposure, others retain antigenic reactivity but show physicochemical alterations after DNA-ase treatment, and still others appear unaffected by DNA-ase. This suggests to them the possibility of at least three antigenic components: those in which antigenic specificity is controlled by DNA, those in which DNA is associated with antigens of a different chemical nature, and DNA-free antigens.

Olitzki, et al (1959) inoculating white mice with Brucella abortus DNA prepared according to the method of Braun et al (1957) demonstrated an excellent immunizing effect by protecting the mice against Brucella abortus infections. Although DNA is not antigenically identical with the surface antigens which immunize mice and produce protecting antibodies, it seems that the association of this antigen with DNA

constitutes a highly active antigen complex which affords excellent immunity of mice against Brucella abortus infections, according to these investigators.

Ultracentrifuge studies by Plescia et al (1961) revealed that the 0.5 per cent phenol extracted DNA preparation contained 25 per cent protein and that of the remaining 75 per cent nucleic acid, 5 to 10 per cent was RNA. This means that only about 70 per cent DNA was in the preparation rather than 80 per cent as originally analyzed.

Differential ultracentrifugation using a Spinco ultracentrifuge with a type 40 rotor, indicated that protein was found in the sediment after centrifugation of 10,000 rpm (10 SED) for 1.5 hours. Most of the nucleic acid was found in the supernate after centrifugation of 40,000 rpm (40 SUP).

Following the immunization schedule of Phillips et al (1957), the antisera obtained were tested against the various ultracentrifuge fractions. The 10 SED protein rich fraction was non reactive. The 30 SED fraction containing less than 2 per cent protein, produced Feulgen positive precipitin bands in agar. This indicated that fraction 30 SED contained most of the reactive antigenic components.

Next, these workers immunized rabbits with ultracentrifuge fractions following the same experimental procedures of Phillips et al (1957). The antisera obtained was reacted against the various antigenic fractions by gel diffusion techniques. The 30 SED fraction antisera produced no visible precipitins as contrasted to the 10 SED fraction antisera which produced very good Feulgen positive precipitin bands.

The non reactive 10 SED fraction was capable of stimulating the formation of precipitating antibodies. On the other hand, the reactive

30 SED fraction was unable to stimulate the production of precipitating antibodies. Failure of this fraction to elicit antibodies is consistent with the results of others who could not detect antibodies in the sera of animals injected with purified DNA. It was concluded that the phenol extracted Brucella DNA was not a simple antigen, but a complex antigen composed of an immunizing fraction 10 SED, and a reactive fraction 30 SED. This suggests that with the attached protein, DNA is a complete antigen which possesses antigenic specificity.

Plescia et al (1963) reported that the precipitin band formed during the gel diffusion analysis of Brucella abortus DNA with rabbit antiserum was Feulgen positive indicating the probable presence of DNA in the precipitate. This data does not mean that DNA contributes the actual determinant group. The DNA might simply have been required for the precipitation of an antigen-antibody complex because of its weight or other physical properties.

In a recent publication, Wilson et al (1965) reported that antibodies formed against a DNA preparation were produced in response to the contaminants alone. Injection of DNA into animals and subsequent serologic analysis can be used for determining the purity of this substance. Wilson (1966) further reported that results obtained in his laboratory were inconsistant with reports of Braun et al (1957) and Phillips et al (1958).

MATERIALS AND METHODS

A. The Organism:

A laboratory strain of Brucella abortus, American Type Culture Collection 7705, Alice G. Evans, NIH strain 456, M. C. G. MM&PH designation G-1, was obtained from the Department of Medical Microbiology and Public Health, Medical College of Georgia. Brucella abortus G-1 was the strain used exclusively in the studies reported here.

The stock culture, lyophilized previously on glass beads, was planted on a tryptose agar slant and incubated at 37° C in a candle jar. After 48 hours incubation, a subculture was streaked on a tryptose agar plate to obtain isolated colonies. Colonies appeared within 48 hours of incubation at 37° C. A single, well isolated colony was picked and streaked on a tryptose agar slant. After growth appeared on the slant, the culture was stored at 4° C. This was the stock culture used for all subsequent inoculations.

Brucella abortus G-1 was studied by standard pure culture methods to assure that its characteristics corresponded to those of the type species as described in Bergey's manual of determinative bacteriology (Breed, 1957). Morphology, Gram stain, colony formation, catalase and urease production, dye sensitivity, and sugar fermentations were determined.

Thimann (1963) indicated that the greatest amount of DNA relative to other constituents of a bacterial cell would be present during the

logarithmic phase of growth. Consequently, the late log phase of the growth curve was chosen as the time for harvesting the cells used in preparation of Brucella antigen.

The growth curve was determined by the method of Gibby and Gibby (1964). Brucella abortus G-1 was inoculated on tryptose agar and incubated at 37° C. Viable cell counts were made at regular intervals over a 72 hour period. The cells were harvested by washing the agar with 10 ml of a 1:1 mixture of normal saline and tryptose broth and placed in chilled (50° C) dilution blanks. Diluted cells were counted by the pour plate method of Lamanna and Mallette (1959). One milliliter of diluted cells mixed with 20 ml of melted tryptose agar (45° C) was poured into sterile petri dishes and allowed to harden. After incubation at 37° C for 48 hours, the total colonies on each plate were counted. Viable cells were considered as those cells which would give rise to a single colony.

B. Preparation of Brucella antigen:

Brucella antigen was prepared according to the procedures of Braun, Burrous and Phillips (1957). Brucella abortus G-1 was grown on tryptose agar for 33 hours at 37° C. Thimann (1963) reported that the best time for harvesting the organism was late in the logarithmic stage of growth. Growth curve determinations showed that this time was 33 hours after inoculation.

The bacteria were harvested with 0.1 M sodium citrate-sodium chloride and treated with melted phenol to give a final concentration of 0.5 per cent. An equal volume of 4 mm glass beads was added and the mixture was shaken in a shaking incubator at 37° C for 48 hours to rupture the cells.

The solution was poured through a wire screen to remove the beads. The beads were then washed once with 0.1 M citrate-saline which was added to the phenol preparation.

The material was deproteinized by treating with chilled 0.3 per cent chloroform and 0.1 per cent amyl alcohol and shaken for 20 minutes in an ice bath. To remove precipitated protein, the mixture was centrifuged at 2000 x g for 20 minutes at 5° C. The supernate was decanted and treated with chloroform and amyl alcohol. Deproteinization procedures were repeated until no precipitate appeared with the addition of chloroform.

Chilled 95 per cent ethanol was added slowly and the mixture was stirred with a glass rod until a fibrous precipitate, macroscopically indicative of DNA, appeared. This precipitate was dissolved in distilled water. The solution was shell frozen and lyophilized at -60° C under a vacuum of 5 u of mercury for 48 hours. A dry powder was obtained. Aliquots of the dried extract were sealed under a vacuum of 5 u mercury and stored at -20° C.

C. DNA Analysis of Phenol Extracted Brucella abortus:

Total nucleic acid content of the extract was determined by dissolving a standard quantity of the extract in saline and determining per cent transmission at 260 mu. The Dische-Stumpf reaction used by Braun et al (1957) and the diphenylamine reaction were two methods used in analyzing specifically for DNA.

The Dische-Stumpf reaction was performed as described by Stumpf (1947). Commercially prepared DNA (Nutritional Biochemical Co.) was used as the

standard. One half milliliter of standard or extract was mixed with 0.5 ml cysteine hydrochloride and 5 ml of sulfuric acid. The DNA content was determined from a standard reference curve by per cent transmission at 490 mu.

DNA was also determined by the diphenylamine color reaction of Burton (1956). Purified diphenylamine dissolved in glacial acetic acid was the color reagent and calf thymus DNA (1.15 mg/ml containing 87.4 ug DNA-P) was used as the DNA standard. Per cent transmission of the DNA standard at 600 mu was plotted against concentration. Total DNA content of the extract was determined by comparison to the reference curve.

Nitrogen content was analyzed according to the procedure of Miller and Miller (1948). Two milliliter aliquots of Brucella antigen were pipetted into micro-Kjeldahl flasks heated, and subsequently followed by treatment with 30 per cent hydrogen peroxide. Nitrogen standards containing 100 mg/ml were prepared from ammonium sulfate. The unknown sample and the standards were treated with Nessler's reagent for color development. Nitrogen content was determined by per cent transmission at 450 mu from a standard reference curve.

Phosphorous content was determined by the method of Fiske and Subbarow (1925). Three milliliters of the extract were pipetted into ignition tubes containing 2.5 ml of 5 N sulfuric acid. The solution was concentrated by boiling, digested with nitric acid, and diluted to 10 ml. Phosphorous standards containing 10 mg/ml were prepared from monobasic potassium phosphate. Color development occured by the addition of 1 ml molybdate and 0.4 ml of 1 per cent Elon (Kodak) in 3 per cent sodium bisulfite. DNA phosphorous was determined from a standard reference curve by per cent transmission at 660 mu.

D. Biochemical Analysis of Brucella antigen:

1. Protein: The lyophilized extract was reconstituted in distilled water to contain 10 mg/ml and diluted serially for spectrophotometric analysis. Protein content was determined by per cent transmission of the extract at 280 mu.

The Folin-Ciocalteau procedure (Lowry et al, 1951) was used to quantitate the protein present in Brucella antigen. Egg albumin containing 200 ug/ml of protein was used as the standard. To 1 ml of sample and standards, 5 ml of alkaline copper sulfate was added. After standing 10 minutes at room temperature 0.5 ml Folin phenol reagent was added. Color developed within 30 minutes and protein was determined from a standard reference curve by per cent transmission at 500 mu.

A check of the protein content was performed with the Biuret test (Gornall et al, 1949). The protein standard was egg albumin containing 200 ug/ml of protein. To 0.5 ml of sample and standards, 9.5 ml of 22.6 per cent sodium sulfate was added. Two milliliters of each solution was then mixed with 8 ml of biuret reagent. After allowing 30 minutes for color development at room temperature, total protein content was quantitated by per cent transmission at 540 mu from a standard reference curve.

2. Lipid: Lipid content of the extract was determined by the Folch procedure (1957). Dried extract was homogenized with a 2:1 chloroform-methanol mixture and filtered on a fritted glass filter. The filtrate was washed three times with water, each time the aqueous phase separated from the organic phase. The aqueous phase was concentrated to dryness. Per cent lipid was determined gravametrically from the amount of lipid material extracted.

3. Polysaccharide: The Anthrone reaction described by Graham and Smydzuk (1965) using a glucose as the standard was employed to ascertain the polysaccharide content of Brucella antigen. Anthrone reagent was pipetted into test tubes chilled to -15° C in an alcohol ice bath. Standards and samples were layered on the reagent. After standing for 15 minutes in the alcohol ice bath, the tubes were shaken to insure thorough mixing of the contents and then placed in a 90° C water bath for 20 minutes. The tubes were cooled by immersion into the ice bath for 1 minute and then placed at room temperature. After allowing 30 minutes for color development, polysaccharide content was determined from a glucose standard reference curve by per cent transmission at 620 mu. A glycogen standard was prepared in order to check for complete hydrolysis of a known polysaccharide.

4. Inorganic Salts: An assay of the inorganic material was undertaken because various stages in the preparation of Brucella antigen required treatment with inorganic salts. Five hundred milligrams of antigen reconstituted in distilled water to 20 mg/ml was dialyzed against 4 liters of glass distilled water at 4° C for 24 hours. The non dialyzable portion was lyophilized at 60° C under a vacuum of 5 u of mercury and then weighed. Per cent inorganic salts was determined from weight lost during dialysis.

In addition, 250 mg of extract was ignited in a tared porcelain crucible on the ignition burner for 6 hours to destroy all organic material. The crucible was cooled to room temperature in a desicator and weighed. Inorganic material was determined from the per cent ash remaining in the crucible.

5. Water: Brucella antigen was lyophilized until a dry powder was obtained. The end point of lyophilization was determined by visual inspection of the material, and as such, the possibility existed that all of the unbound water was not removed from the extract. Free water of the lyophilized extract was determined by drying the material. One hundred milligrams of antigen obtained from a sealed vial was dried at 110° C under a vacuum for 3 hours. The material was weighed again and the per cent water was determined by weight lost. Drying the extract at elevated temperatures might have decomposed part of the material. As a check of the drying procedure, 100 mg of Brucella antigen obtained from a sealed vial was relyophilized under a vacuum of 5 u of mercury at -60° C for an additional 48 hours and then weighed. Per cent water was determined by weight loss.

E. Animals:

Ten male White Rock chickens were obtained from Dr. Ross Brown in Andersonville, Georgia, and two male White Rocks were obtained from Southeastern Hatchery in Leesville, South Carolina. These roosters were all five to six months old and weighed approximately 4.5 kilograms each. The roosters were given normal care by the Medical College of Georgia Veterinary Services. Leg bands were placed on the leg of each chicken as a means of identification and the serum obtained from each chicken was numbered according to the leg band.

Ten young adult male albino guinea pigs weighing approximately 350 grams each were obtained from Mamie Gardner Laboratory Animals, Charlotte, North Carolina. These guinea pigs were given normal care by the Medical College of Georgia Veterinary Services.

F. Immunization:

Ten chickens received injections of the material extracted from Brucella abortus G-1. These injections consisted of 2 ml of dissolved antigen and Freund's adjuvant complete (Difco) in a 1:1 ratio. A total of eight injections were administered to each animal. Two control chickens received eight 2 ml injections of a 1:1 Freund adjuvant-saline mixture. The immunization schedule is found in Table I.

The animals were bled by cardiac puncture prior to immunization and on day 25, which was 8 days following the final injection. Chicken serum contains a large amount of lipids which may interfere with serological reactions (Campbell et al, 1964). Lipid concentration was minimized by starving the chickens 24 hours prior to bleeding. The serum was clarified of the remaining lipids by centrifugation at 15,000 rpm for 30 minutes at 0° C. The serum was separated from the solidified lipid material.

TABLE I

SCHEDULE FOR IMMUNIZING CHICKENS WITH PHENOL EXTRACTED BRUCELLA ANTIGEN.

Injection number	Day	mg Sample/ injection
1	1	2.0
2	3	2.0
3	5	3.0
4	8	4.0
5	10	6.0
6	12	8.0
7	15	12.0
8	17	15.0
	Total amount injected	52.0 mg

G. Serological Analysis:

Ring Precipitin Tests:

Each serum sample was tested for precipitating antibodies by the ring precipitin test performed by standard procedures. In all cases the antiserum was placed in the bottom of 100 mm x 3 mm precipitin tube and the antigen was layered on top. Tests were carried out at room temperature and observed after 15 minutes. Initial testing was set up diluting the serum 1:1 in physiological saline. End point precipitin titers were obtained on all positive serum samples. The antigen was prepared by dissolving 100 mg of Brucella antigen in 10 ml of distilled water and clarifying the solution by centrifugation at 25,000 x g for 30 minutes. The antigen was serially diluted to determine the lowest concentration which would give a positive reaction with antiserum diluted 1:1.

Goodman et al (1951) reported that maximum precipitation of a positive reaction occurs in chicken serum with a saline concentration of 8 to 13 per cent. Ring tests were performed on all serum samples using various salt concentrations to determine the optimum saline concentration required for precipitation. The serum was diluted 1:10 in 0.85, 8, 10 and 13 per cent borate buffered saline and Brucella antigen in a concentration of 10 mg/ml was layered over the serum in precipitin tubes.

Experiments were designed to determine if antibodies were produced in response to DNA. Preliminary tests indicated that herring sperm DNA with pH 4.5 precipitated serum protein nonspecifically. Therefore this DNA was adjusted to pH 7.2 with 1 N sodium hydroxide. Ring precipitin tests were performed by reacting chicken antiserum diluted 1:1 with 0.5 per cent sperm DNA adjusted to pH 7.2.

Since Brucella abortus G-1 was grown on tryptose agar, the possibility existed that chicken antiserum might have antibodies produced in response to tryptose. Ring precipitin tests were set up using chicken serum diluted 1:1 and 1 per cent tryptose as the antigen.

Gel Diffusion:

Preer tube double diffusion was set up as described by Campbell et al (1964). Serum diluted 1:1 in saline was mixed with an equal volume of 1.2 per cent Special Agar Noble (Difco) and placed as the bottom layer in 100 mm x 3 mm gel diffusion tubes. After the mixture hardened, 0.6 per cent agar nobel was placed as the intermediate layer. Brucella antigen containing 10 mg/ml was added to the top layer in the Preer tubes. The tubes were incubated at room temperature and observed after 12 hours.

Ouchterlony gel diffusion plates were prepared by pouring 20 ml of 1 per cent Agar Noble containing 1:10,000 merthiolate into petri dishes. Wells were cut in the agar and the bottom of these wells were sealed with 1 drop of agar. Serum diluted 1:1 was placed in the center well and Brucella antigen in concentrations varying from 40 ug/ml to 2.5 ug/ml were placed in the peripheral wells. After 48 hours incubation at room temperature, serum and antigen were again added to their respective wells. Attempts to force the antigen to diffuse through the agar in certain plates were attempted by filling the antigen wells twice a day for 2 days and then adding antiserum on the third day.

Feulgen Reaction:

Preliminary tests show that the Feulgen reagent stains agar making

DNA identification difficult when DNA is incorporated in the agar. Modifications of the Feulgen procedure were set up. Several plates with DNA incorporated in agar were treated with 1 N hydrochloric acid at 50° C for 5, 10 and 15 minutes intervals followed by exposure to the Feulgen reagent for 0.5, 1 and 2 hours. The modified Feulgen reaction used in these studies is as follows: The agar was treated with 1 N hydrochloric acid at 50° C for 5 minutes, stained with Feulgen reagent for 2 hours, rinsed with sulfurous acid and washed with tap water.

In order to determine if DNA could be detected in agar, 1.0, 0.1, 0.01, 0.001 and 0.0001 per cent increments of sperm DNA were placed in wells of Ouchterlony plates and allowed to diffuse. These plates were stained with the modified Feulgen procedure. Ouchterlony gel diffusion plates which contained a positive precipitin band were also stained by the modified Feulgen reaction to ascertain if DNA was present in the precipitate.

Agglutination Tests:

Microscopic slide agglutination tests were performed according to procedures described by Lederle Laboratories. A 0.2 ml pipette was used to deliver chicken sera diluted 1:10 in saline in 0.08, 0.04, 0.02, 0.01, 0.005 and 0.002 ml quantities onto clean glass slides. Using a standardized dropper, one drop (0.03 ml) of Brucella abortus febrile antigen (Lederle Laboratories) was added to the varying quantities of serum. The serum and antigen were mixed with a wooden applicator stick and the slides were tilted back and forth. Agglutination which occured within 3 minutes was considered a positive reaction.

Tube agglutination tests described by Lederle Laboratories were also

performed using chicken sera and the febrile antigen. Each serum was diluted serially in physiological saline so that a final volume of 0.5 ml of diluted serum was obtained. Pre-immunization sera and sera from control chickens served as controls. One half milliliter of Brucella abortus febrile antigen diluted 1:100 in saline was added to each tube. As an antigen control, 0.5 ml of diluted febrile antigen was mixed with 0.5 ml of saline. The tubes were incubated at 37° C and observed after 24 hours.

Absorption of Antiserum:

One tenth milliliter of chicken antiserum was mixed with 0.3 ml of Brucella abortus febrile antigen and incubated at 37° C for 4 hours. After the antigen was removed by centrifugation at 1000 x g for 15 minutes, 0.6 ml of 12 per cent saline was added to the serum for a final concentration of 1:10.

Agglutination tests using the febrile antigen were performed in order to insure complete removal of antibodies to the somatic antigen of Brucella abortus. Ring precipitin tests were then set up testing the absorbed serum with Brucella antigen. Unabsorbed serum diluted 1:10 in 12 per cent saline was used as the controls.

Extraction of Polysaccharides:

Brucella abortus G-1 was harvested with saline after 48 hours growth and concentrated by centrifugation. Methods described by Oser (1961) were used for extraction of polysaccharide. The cells were treated with 30 ml of 30 per cent potassium hydroxide and the mixture was placed

in a boiling water bath for 1 hour. The solution was cooled and clarified by centrifugation. Ninty five per cent ethanol was added to precipitate the polysaccharide. The precipitate was washed once with ethanol and once with diethyl ether and then dissolved in 15 ml of distilled water.

The Anthrone reaction was performed to quantitate the extracted polysaccharide. Ring precipitin tests were set up using immune chicken serum and the polysaccharide as antigen in order to determine whether or not antibodies in the sera were directed toward the polysaccharide.

Enzymatic Treatment of Brucella Antigen:

Brucella antigen was treated with trypsin (1:250, Nutritional Biochemicals) according to the method of Phillips et al (1958). The mixture, which contained an antigen concentration of 50 ug/ml of DNA and a trypsin concentration of 0.03 per cent, was incubated in a 37° C water bath for 1.5 hours. At the end of the incubation period, the solution was chilled at 4° C in an ice bath to retard further enzyme activity. Ring precipitin tests were set up using chicken antiserum and trypsin treated antigen.

The extract was also treated with deoxyribonuclease (30,000 dornase units per mg., Nutritional Biochemicals) according to the procedure of Phillips et al (1958). Brucella antigen was mixed with DNAase so that a final concentration of 50 ug/ml of DNA and 0.03 per cent DNAase was obtained. The solution was incubated at 37° C for 1.5 hours and then chilled to 4° C. DNAase treated as well as untreated extract are tested with chicken antiserum by the ring precipitin test.

Passive Hemagglutination:

Passive hemagglutination was attempted by the method of Stavitsky (1958). One aliquot of human O cells was coated with Brucella antigen and a second aliquot was coated with herring sperm DNA. Antigen coated erythrocytes were added to inactivated chicken serum samples which were previously absorbed with both human O cells and tanned human O cells. Diluent, cell and serum controls were set up.

The Dische-Stumpf reaction was performed on the coated erythrocytes to detect the presence of DNA. Both coated cells and normal cells were treated with cysteine hydrochloride and sulfuric acid and the per cent transmission was determined at 490 mu. This test was a qualitative determination of DNA and quantitative standards were not set up.

Reverse hemagglutination was performed according to the method of Boyden (1958) in order to determine the applicability of hemagglutination for this study.

Passive Cutaneous Anaphylaxis:

Demonstration of antibodies to Brucella antigen and to a commerically prepared DNA was attempted by passive cutaneous anaphylaxis according to the method of Ovary (1948). Chicken serum samples were injected in 0.05 ml increments intracutaneously into the shaven backs of albino guinea pigs. Fifteen hours later, 0.5 ml of antigen mixed with 1.0 ml of Evan's blue dye (Warner) was injected into the heart of each guinea pig.

Injection sites were observed for 1 hour for a marked blue coloring of the injected area. The animals were then sacrificed and their skins removed. The subcutaneous surface of the skin was examined for evidence of the blue dye.

RESULTS

The Organism:

The organism is a gram negative, non-motile, asporogenous, coccobacillus less than 1 u in length and about 0.25 u wide. On tryptose agar, small convex, smooth, translucent colonies appeared within 48 hours. This culture does not require an increased carbon dioxide environment, but growth is slightly enhanced when one is provided. Biochemically, the organism does not ferment glucose, sucrose, lactose, maltose, or mannose. This strain is both catalase and urease positive. The organism is inhibited by 1:800 dilution of thionine and grows in the presence of a 1:200 dilution of basic fuchsin. All of these tests confirm that the stock culture is Brucella abortus (Breed, 1957).

The Growth Curve:

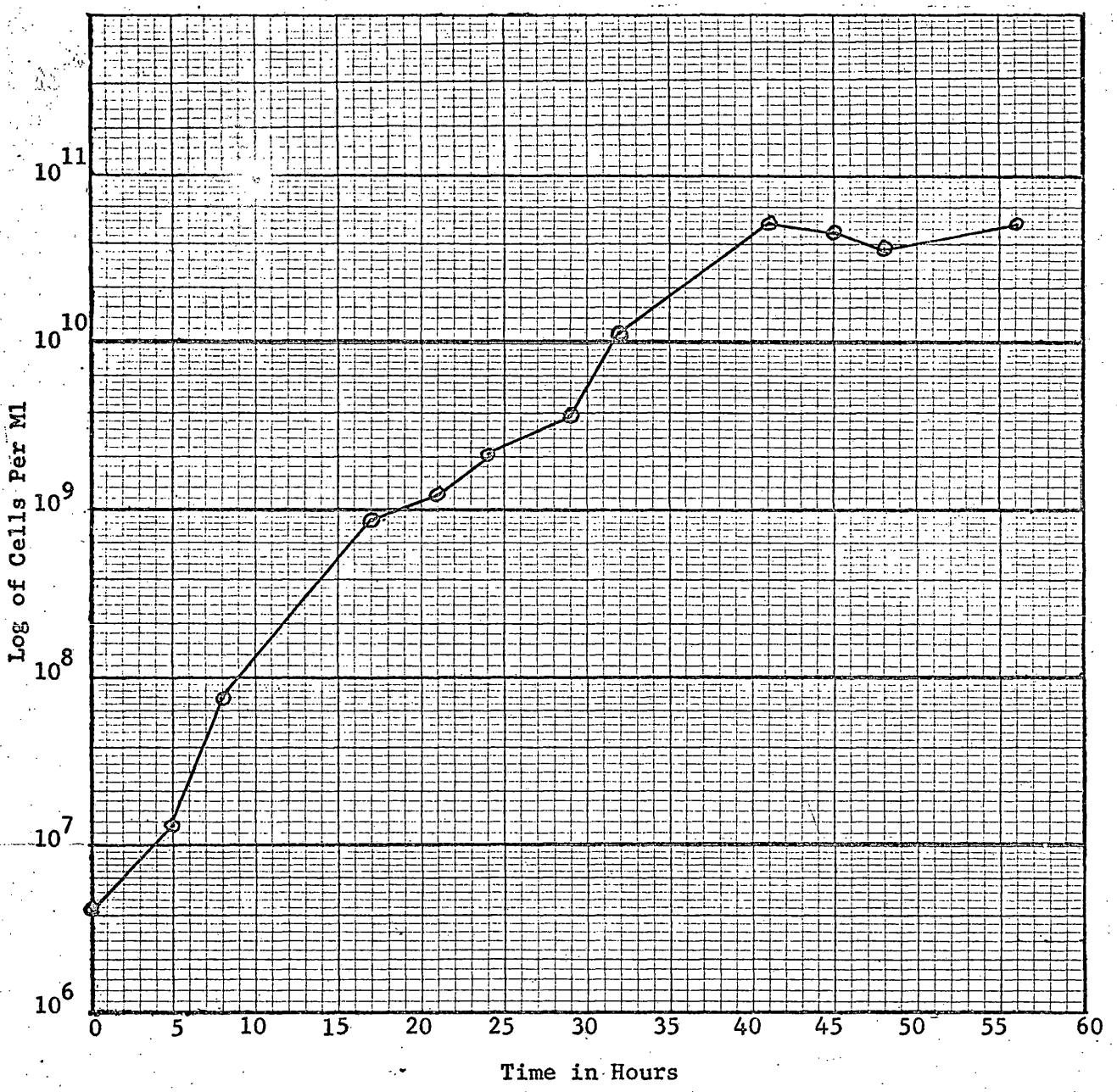
A growth curve was prepared by counting viable cells incubated at 37° C for various increments of time. Figure I is a graph of the logarithm of viable cells per milliliter plotted against incubation time in hours. From these data and from reports of Thimann (1963), that the late logarithmic stage of growth would be best for DNA extraction, all cells used to prepare the antigen in this study were harvested after an incubation of 33 hours.

Biochemical Analysis:

Table II represents an outline of the chemical analysis of Brucella

FIGURE 1

Growth Curve of Brucella abortus G-1 Grown on Tryptose Agar



Time in Hours	Cells Per Ml
0	5.50×10^{6}
5	1.96×10^{7}
8	8.30×10^{7}
17	9.20×10^{8}
21	1.49×10^{9}
24	2.69×10^{9}
29	4.75×10^{9}
32	1.34×10^{10}
41	6.35×10^{10}
45	5.58×10^{10}
48	4.67×10^{10}
56	6.08×10^{10}

antigen. The following data describe in detail all results obtained from the analysis.

1. DNA: Diphenylamine and spectrophotometric analysis at 260 mu has shown that each mg of Brucella antigen contains 40 ug of DNA. This comprises 4 per cent of the dry weight. The Dische-Stumpf reaction revealed that 500 ug of 5 per cent of 10 mg of extract was DNA. Nitrogen phosphorous mole ratio of Brucella antigen had a range of 4.08-4.13. The extinction coefficient at 260 mu was 7,900-8,000.

2. Protein: In 25 mg of extract, 60 ug of protein was determined by the Folin-Ciocalteau method, by the Biuret test and by spectrophotometric analysis at 280 mu. Per cent protein of the extract is 0.25 per cent.

3. Lipid: Determinations using the Folch procedure has revealed that 0.4984 g of extract contains 0.0054 g or 1.08 per cent lipid material.

4. Polysaccharide: The Anthrone reaction revealed that 6.667 mg of extract contains 375 ug of polysaccharide. It is calculated that 5.62 per cent of the material is polysaccharide. The reliability of this reaction was checked by a comparison of a known amount of glycogen and the glucose standard. Fifty micrograms of glycogen was shown to contain 50 ug of glucose by the Anthrone test.

5. Inorganic salt: To test for salts, 0.5000 g of Brucella antigen was dialyzed against distilled water. After dialysis, the nondialyzable portion was lyophilized and found to weigh 0.2374 g. It is calculated that 0.2626 g or 52.52 per cent is dialyzable. The ignition of 0.2500 g of extract produced 0.1132 g of ash. Per cent ash of the total extract is 45.28 per cent.

6. Water: Heating 0.0945 g of antigen to 110° C, 0.0282 g or

29.84 per cent of the material was evaporated. Relyophilization of 0.0734 g of extract for 48 hours revealed that 0.0249 g or 33.92 per cent is water.

In Table III a summary of the composite quantities of Brucella antigen is depicted. It can be seen from these data that inorganic salts and water comprise 80 per cent of the extract. Polysaccharides constitute 5.6 per cent while only 4.5 per cent of the material is DNA. The antigen also contains 1 per cent lipid and 0.25 per cent protein. Percentages reported represent the averages obtained from results of all procedures.

TABLE II

BIOCHEMICAL ANALYSIS OF PHENOL EXTRACTED *BRUCELLA* ANTIGEN.

Material	Test	Milligrams/Gram of Antigen
DNA	1. Absorbance at 260 mu	40.0
	2. Diphenylamine	40.0
	3. Dische-Stumpf	50.0
Protein	1. Absorbance at 280 mu	2.5
	2. Folin-Ciocalteau	2.5
	3. Biuret	2.5
Lipid	Folch	10.8
Polysaccharide	Anthrone	56.2
Inorganic salts	1. Dialysis	525.2
	2. Ash content	452.8
Water	1. Heating to 110° C	298.4
	2. Relyophilization	339.2

TABLE III

COMPOSITE QUANTITIES OF MATERIAL FOUND IN *BRUCELLA* ANTIGEN

Material	Per cent of Total
DNA	4.5
Protein	0.3
Lipid	1.1
Polysaccharide	5.6
Inorganic salts	48.9
Water	31.9
MATERIAL ACCOUNTED FOR	92.3
UNACCOUNTABLE MATERIAL	7.7

Ring Precipitin Studies:

The ring precipitin test was found to be satisfactory for the detection of antibodies in chicken serum to phenol extracted Brucella antigen. Six serum samples, post immunization sera 716, 719, 747, 769, 770 and 774 diluted 1:1 in physiological saline, produced a precipitin ring when overlayed with Brucella antigen in concentration of 10 mg/ml. All pre-immunization serum samples and serum from control chickens were negative. Saline controls substituted for serum and/or antigen were negative.

Serum titers were obtained by diluting positive sera 1:10 in saline and diluting the sera two fold until end points were reached starting with an antigen in concentration of 10 mg/ml. Serum titers reported in Table V ranged from 1:40 to 1:160. The antigen was also diluted serially to determine the minimum concentration required to give a positive reaction with antiserum diluted 1:1 in saline. Table IV shows that minimum antigen concentrations required for precipitation ranged from 125 ug/ml to 500 ug/ml. There does not seem to be a correlation between high serum titers and minimal antigen concentrations in the ring precipitin test.

In testing for the optimum salt concentration of chicken serum necessary for precipitation, positive reactions occured with 8, 10 and 13 per cent saline. Only those sera which produced precipitates in physiological saline, were positive and it was noted that higher concentrations of salt resulted in a slight increase in precipitation. No precipitation occured with pre-immunization serum nor in control sera with increased salt concentrations when tested with the antigen.

TABLE IV

RING PRECIPITIN TEST USING CHICKEN SERUM AND *BRUCELLA* ANTIGEN

Serum Sample	Serum titer	Minimum antigen concentration required for precipitation
705	-	-
716	1:40	500 ug/ml
717	-	-
719	1:80	250 ug/ml
721	-	-
747	1:160	250 ug/ml
762	-	-
769	1:320	250 ug/ml
770	1:160	125 ug/ml
774	1:160	500 ug/ml
761*	-	-
780*	-	-

* Serum from control chickens receiving injections of adjuvant and saline.

Ring Precipitin tests reacting Chicken serum with either tryptose or sperm DNA as antigen produced negative results.

Using polysaccharide extracted from Brucella abortus G-1 as antigen, 4 serum samples, post immunization sera 747, 769, 770 and 774 produced a positive ring precipitin. Controls were prepared by using serum from uninoculated chickens as well as pre-immunization sera. All controls were negative. Analysis of the antigen by the anthrone reaction indicates that the polysaccharide concentration of this crude extract is 7.3 mg/ml. As a comparison, the concentration of Brucella antigen used as antigen in the ring test is 10 mg/ml and 5.6 per cent or 560 ug/ml is analyzed as polysaccharide material.

Absorption of Chicken Antiserum with Brucella abortus Febrile Antigen:

Post immunization serum samples 716, 719, 747, 769, 770 and 774 which produce positive precipitin and positive agglutinin reactions were absorbed with Brucella abortus febrile antigen. Microscopic slide tests were set up reacting the absorbed sera with the febrile antigen. Unabsorbed sera agglutinated the antigen whereas the absorbed sera showed no positive agglutination. In ring precipitin tests using Brucella antigen in concentration of 10 mg/ml, unabsorbed serum produced a positive reaction and the absorbed serum was negative. Absorbtion of the chicken antiserum with Brucella abortus febrile antigen removed all antibodies to the particulate antigen as determined by the agglutination test and to the soluble antigen as determined by the ring precipitin test.

Enzymatic treatment of Brucella Antigen:

Brucella antigen treated with trypsin did not affect the ring

precipitin reaction of five positive serum samples but prevented a positive reaction with serum sample 716. Treatment of the antigen with DNA-ase did not alter the reactivity with positive sera.

Gel Diffusion:

A single diffuse precipitin band occured in Preer double diffusion tubes at the interface between the intermediate agar layer and the antigen. This reaction occured in tubes containing post immunization serum samples 719, 747, 769, 770 and 774. Figure 2 illustrates that Brucella antigen does not migrate well through the agar. It appears that precipitation was the result of serum alone diffusing through the agar and reacting with the extract at the top of the intermediate agar layer. Control tubes were prepared by substituting saline for the antigen in tubes which had a positive precipitation. Control tubes and tubes containing pre-immunization serum or serum from control chickens were negative.

In Ouchterlony double diffusion plates, a single copious precipitation region was observed after 72 hours with post immunization serum samples 716, 719, 769, 770 and 774. Figure 3 shows that precipitation appeared by the inner aspect of the antigen well facing the antiserum. Greatest precipitation occured next to wells containing antigen concentrations of 40 mg/ml and lesser degrees of precipitation were noted with decreasing extract concentrations. Precipitation was not found in pre-immunization sera, with control sera, when saline was substituted for antigen in positive reactions. With both Preer tubes and Ouchterlony plates, it appears that Brucella antigen did not diffuse well through agar.

FIGURE 2

PREER TUBE GEL DIFFUSION

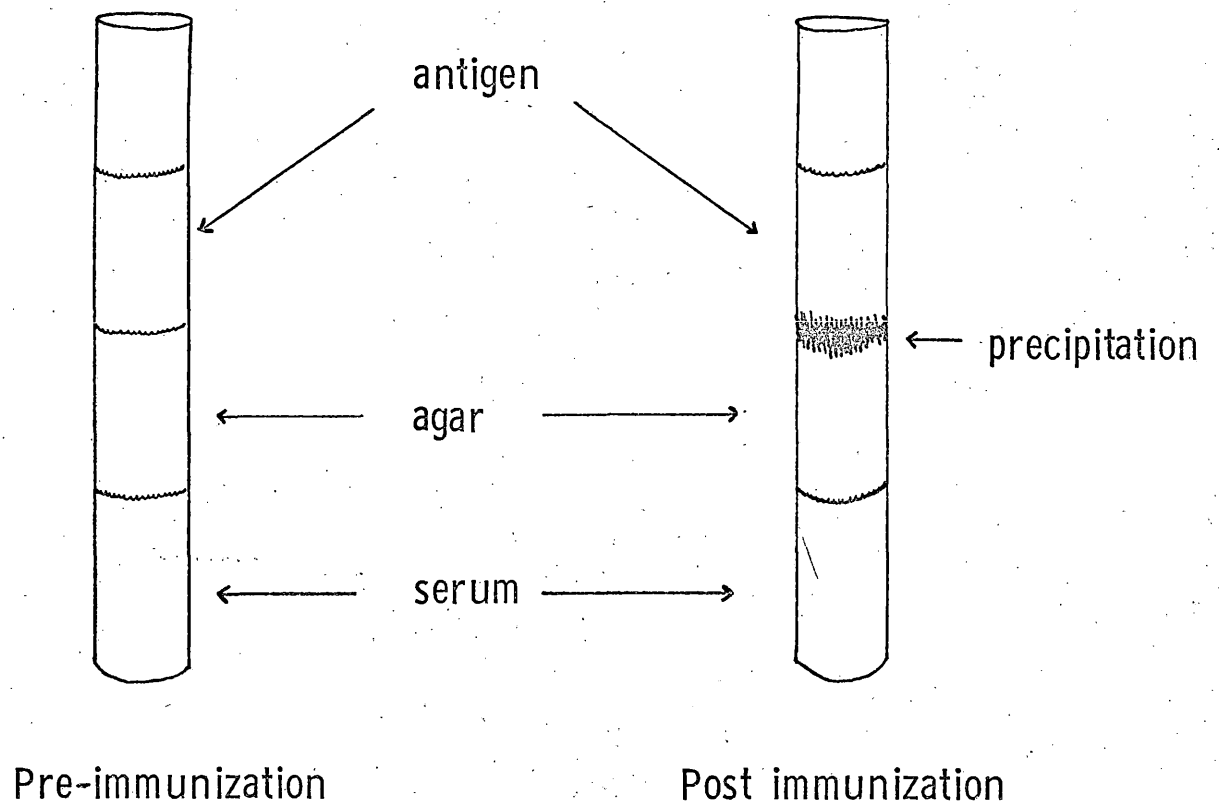
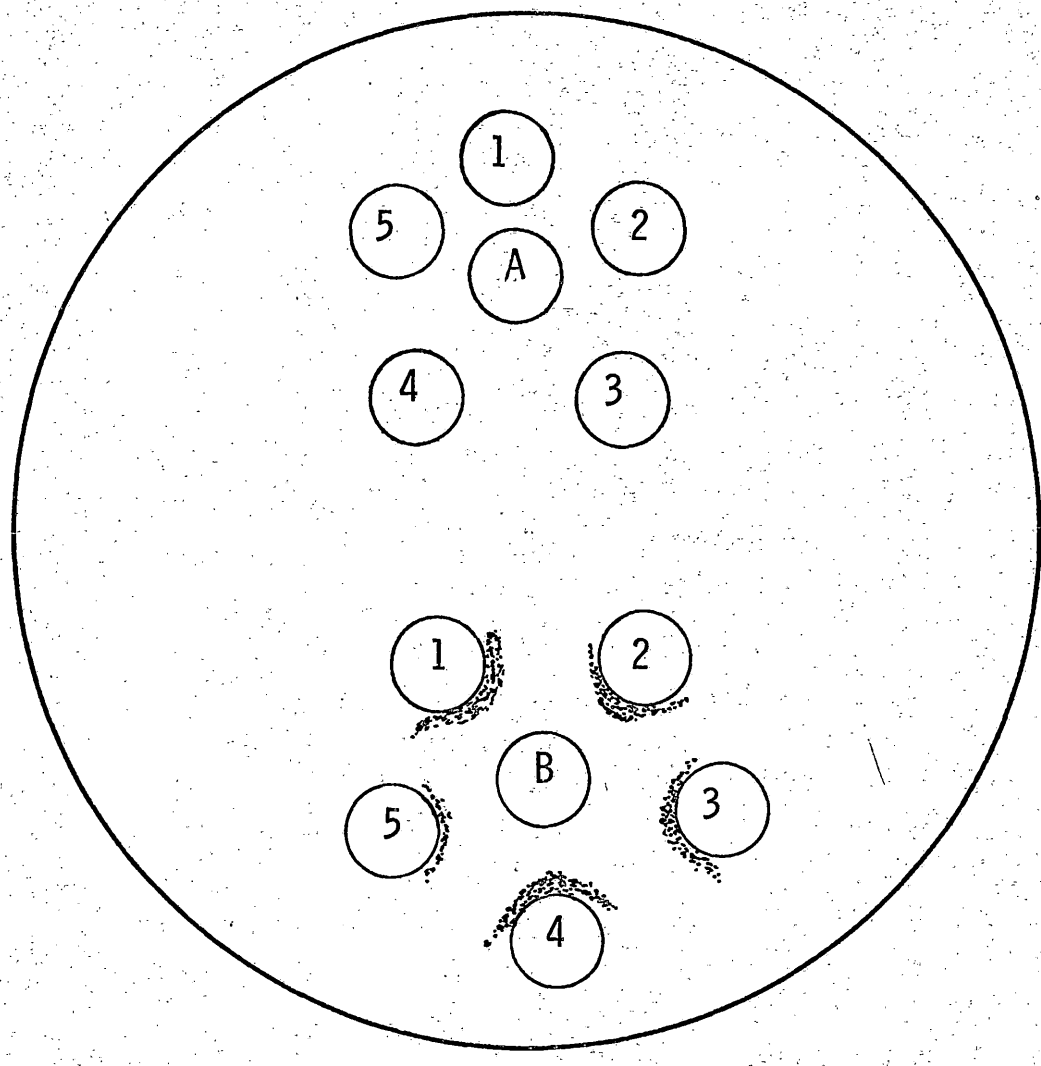


FIGURE 3

OUCHTERLONY GEL DIFFUSION



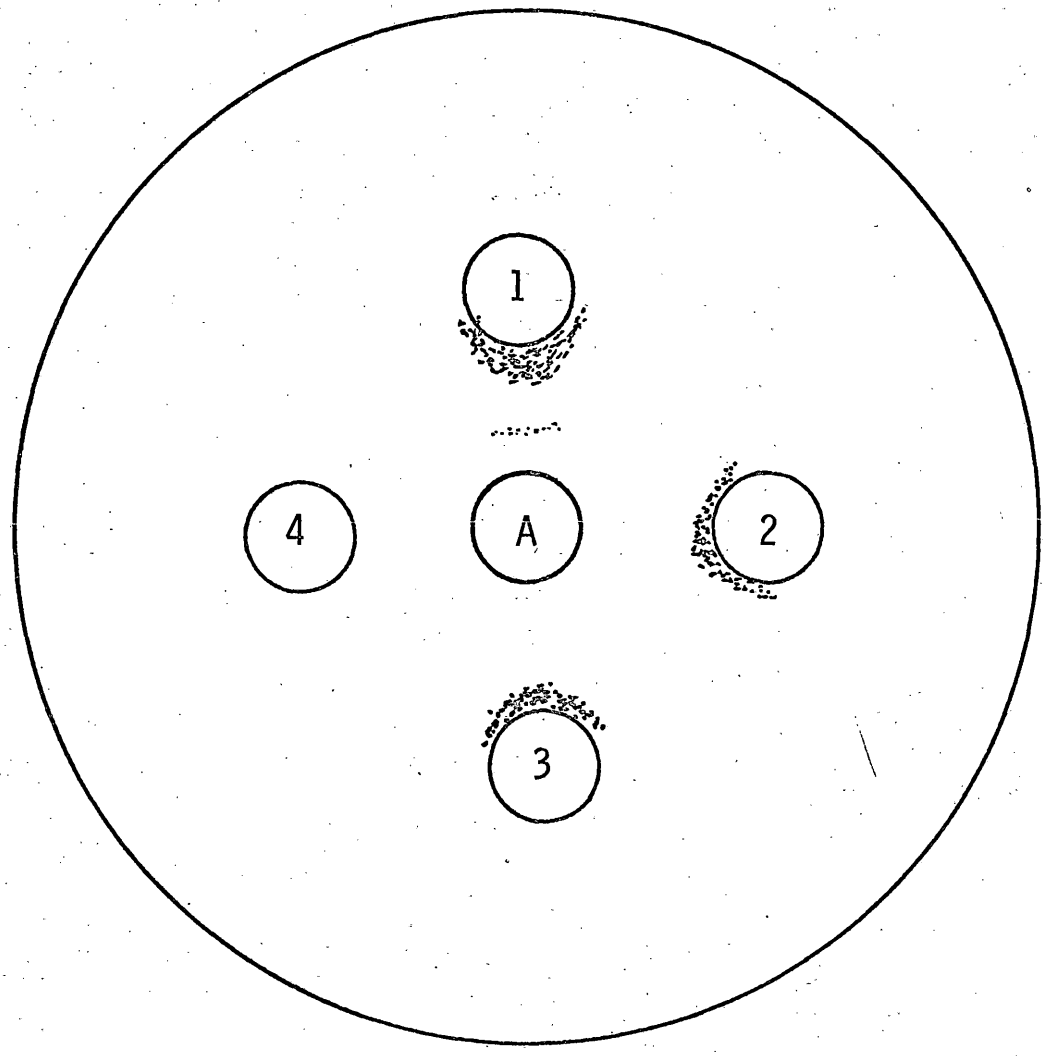
A= pre-immunization serum

B= post immunization serum

1= antigen concentration 40 mg/ml
2= antigen concentration 20 mg/ml
3= antigen concentration 10 mg/ml
4= antigen concentration 5 mg/ml
5= antigen concentration 2.5 mg/ml

FIGURE 4

OUCHTERLONY GEL DIFFUSION



A= Antiserum diluted 1:1 in saline
1= Polysacchaside containing 7.3 µg/ml
2= Brucella antigen containing 10 mg/ml
3= Brucella febrile antigen
4= Ruptured Brucella cells

Feulgen Reaction:

Control plates were set up in which DNA was allowed to diffuse through agar. DNA was specifically stained and the agar was non reactive using the modified Feulgen reaction. As little as 100 ug/ml of DNA diffused through agar could be detected by this method.

Plates having precipitin bands were treated with the Feulgen reagent. None of these precipitin bands stained Feulgen positive. Control plates with DNA diffused through the agar were treated together with the Ouchterlony plates and stained Feulgen positive.

Agglutination Studies:

Post immunization serum samples 716, 719, 747, 769, 770 and 774, diluted 1:1 in saline, agglutinated whole cells of Brucella abortus G-1 in a saline suspension. Pre-immunization sera, serum from control chickens, and physiological saline failed to agglutinate the organism.

Chicken serum was also tested for agglutinating antibodies to Brucella abortus febrile antigen by both the microscopic slide and tube agglutination tests. Serum titers were obtained from the smallest concentration of serum diluted serially in saline which would agglutinate the antigen. It can be seen in Table V that serum titers to the febrile antigen obtained from the microscopic slide agglutination tests ranged from 1:80 to 1:320. In the more sensitive tube agglutination tests, serum titers ranged from 1:160 to 1:2560. Pre-immunization sera, sera from control chickens, and saline all produced negative results. A correlation was noted between the febrile antigen and Brucella abortus G-1 in that both antigens were agglutinated by the same serum samples, post

TABLE V

AGGLUTINATION TEST OF CHICKEN SERUM AND *BRUCELLA ABORTUS* FEBRILE ANTIGEN

Serum Sample	Slide Agglutination Titer	Tube Agglutination Titer
705	-	-
716	1:160	1:320
717	-	-
719	1:80	1:160
721	-	-
747	1:320	1:1280
762	-	-
769	1:320	1:1280
770	1:160	1:640
774	1:320	1:2560
761*	-	-
780*	-	-

* Serum from control chickens receiving injections of adjuvant and saline.

immunization sera 716, 719, 747, 769, 770 and 774. The febrile antigen was prepared from Brucella abortus strain #8, original Huddleson #251 (Hammond, 1966).

Indirect Hemagglutination:

Spontaneous agglutination of human cells occured in all tubes in which chicken serum was added. It appears as though heterologous antibodies in chicken serum to human erythrocytes could not readily be absorbed.

Adsorption of Brucella antigen and DNA on the surface of tannic acid treated red blood cells was demonstrated spectrophotometrically by the method of Stumpf (1947). The appearance of a pink color in the solution when both preparations were treated with cysteine hydrochloride and sulfuric acid indicated that DNA was present.

Passive Cutaneous Anaphylaxis:

The injection sites on the back of the guinea pig were observed for 2 hours after inoculation. A blue coloration at these sites indicative of an antigen-antibody reaction, did not appear. It appears that the animals were properly injected with the antigen-Evan's blue dye mixture because the eyes, nose, ears, mouth, tongue and paws were tinted blue. Examination of the subcutaneous surface of the skin also failed to indicate a positive reaction. Passive cutaneous anaphylaxis to chicken antiserum and Brucella antigen or DNA was not observed in the guinea pig.

DISCUSSION

One of the more important aspects of this research was to ascertain if phenol extracted DNA (Braun et al, 1957) was antigenic in chickens. Biochemical analysis of Brucella antigen has shown that DNA is only a minor component of the extract. Results from three methods of analysis showed that DNA comprises 4 to 5 per cent of the extract. Braun (1965) indicated that this low percentage of DNA in Brucella antigen was a result of the extract being lyophilized. However Chargaff and Davidson (1955) reported that drying DNA does not diminish the quantity obtained nor does it hamper the chemical analysis of this substance. Removal of water does alter the structure of the molecule. RNA was not analyzed per se because determination of the extract by spectrophotometric procedures showed that 4 per cent of the material was nucleic acid. Since the diphenylamine reaction indicated that 4 per cent of this material is DNA, it was concluded that RNA could only be present in minute amounts if at all.

Almost half of the lyophilized material is inorganic salts and about a third of the dry weight is free water. The high salt content is attributed in part to the citrate-saline solution used in extracting the antigen, in part to the inorganic salts in the tryptose agar medium on which the organism was grown and in part to the inorganic constituents of the bacterial cell. An explanation for the large amount of free water in the antigen is that a "dry" extract was obtained after lyophilization for 48 hours and it was assumed that all the water had been removed. Only after

additional drying was more water detected in the extract. Polysaccharides constitute a significant portion of the antigen. This finding is supported by reports of Martinez-Segovia et al (1965) that phenol extracted DNA precipitated by ethanol contains large amounts of polysaccharides. Treatment of the extract with chloroform resulted in the antigen containing only 1 per cent lipids and 0.25 per cent protein. A significant aspect of the biochemical analysis is that more than one procedure was used to quantitate each component of the extract and the results were remarkably consistant.

Evidence is presented in this study that phenol extracted Brucella antigen is antigenic in chickens. Ring precipitin tests were used to demonstrate the antibodies present in chicken antiserum because of the simplicity of design and high degree of accuracy. The ring test has shown that 60 per cent of the injected chickens produced antibodies to Brucella antigen. It should be noted that Plescia et al (1963) reported that only 5 per cent of rabbits injected with Brucella antigen produced detectable "anti-DNA" antibodies in response to this extract. The absence of antibodies reactive with the extract in pre-immunization serum and in the serum of control chickens indicates that precipitins were produced in response to the injected material.

In these studies chicken antiserum agglutinated Brucella abortus G-1. Topley and Wilson (1936) report that normal chickens frequently have agglutinins to Brucella abortus in titers as high as 1:200. In agglutination studies using Brucella febrile antigen, all of the pre immunization and control sera were negative and the positive sera had titers as high as 1:2560. It is therefore concluded that the chickens used in this study had no prior exposure to the Brucella organism detectible by methods utilized here and that any antibodies formed against the bacterium were in response

to the injections of Brucella antigen.

A correlation can be drawn between the ring precipitin test and the agglutination test. All serum samples which produced positive precipitin rings also agglutinated whole cells and the febrile antigen. Antibodies to the whole cell and to the extract could be removed by absorbing the antiserum with the febrile antigen. In addition, Olitzki et al (1959) reported that Brucella antigen as a vaccine protected mice against brucellosis. It appears that antibodies to Brucella antigen were formed against surface component of Brucella abortus and probably not against material derived from the internal structure of the organism.

Antibodies to Brucella antigen were not demonstrated by passive hemagglutination. Sufficient work was not done with this technique and no conclusion could be made. Passive cutaneous anaphylaxis to Brucella antigen has been reported by Miescher et al (1960). However this procedure is inapplicable in demonstrating antibodies present in chicken serum. Halpern (1963) reported that hypersensitivity of chicken serum can not be transferred to the guinea pig.

The use of agar gel techniques has shown that Brucella antigen does not migrate well through agar. In Ouchterlony plates reacting Brucella antigen with chicken antiserum, a diffuse precipitin band appeared adjacent to the well containing antigen. Reacting Brucella febrile antigen with antiserum, one precipitin band also formed adjacent to the antigen well. However utilizing extracted polysaccharide as antigen, two precipitin bands were formed. One band occured next to the antigen and the second band migrated and appeared mid way between the serum and antigen wells. The diffuse precipitin bands occuring adjacent to Brucella antigen, the febrile antigen and the extracted polysaccharide appear to be similar. The

second precipitin band found in the reaction of the extracted polysaccharide and the antiserum may be due to differences in concentration. Brucella antigen contains about 50 per cent inorganic salts and has a polysaccharide concentration of 560 ug/ml whereas the extracted polysaccharide contains 7.3 mg/ml polysaccharide. Crowle (1961) reports that an increase in antigen concentration may cause the formation of a secondary precipitate. Crowle further reports that recharging the agar with antigen may cause a small amount of displacement and this could result in band multiplication.

As best as can be determined, DNA is not involved in this antigen-antibody reaction. Utilizing the Feulgen procedure, as little as one drop of a solution containing 100 ug/ml of sperm DNA can be detected in agar gel. The precipitin bands formed in Ouchterlony plates from a reaction of chicken antiserum and Brucella antigen containing 200 ug/ml DNA did not stain with the Feulgen reagent. One explanation may be that the precipitin band did not contain sufficient DNA to be stained by the Feulgen reagent. Another possibility is that DNA was not present in the precipitate. Sperm DNA would not react with chicken antiserum in the ring test and treatment of the antigen with DNAase had no visible effect on the precipitin reaction.

Hershey et al (1935) reported that polysaccharides of Brucella cell walls are antigenic in rabbits. Biochemical analysis of Brucella antigen has shown that 1 g of extract contains 56.2 mg of polysaccharide material. Polysaccharide extracted from Brucella abortus G-1 produced a positive precipitin reaction with four post immunization serum samples. Reaction of Brucella polysaccharide with chicken antiserum tends to indicate that this material is responsible, at least in part, for the antigenic activity of phenol extracted Brucella antigen.

SUMMARY

Phenol extracts were prepared from Brucella abortus. This material was analyzed according to standard biochemical procedures. The extract was used as antigen and injected into chickens. The sera obtained were studied using serologic methods.

1. Chemical analysis of phenol extracted Brucella abortus has shown that the lyophilized extract contains 4.5 per cent DNA, 0.25 per cent protein, 1 per cent lipid, 5.6 per cent polysaccharide, 49 per cent inorganic salt and 32 per cent water. It has a nitrogen phosphorous mole ratio of 4.08 and an extinction coefficient of 7,900.

2. Antibodies to Brucella antigen were detected in 60 per cent of the chickens immunized. These antibodies were demonstrated in the ring precipitin and agglutination tests. In Ouchterlony gel diffusion studies a single diffuse precipitin band appeared adjacent to antigen wells containing either Brucella antigen or the febrile antigen.

3. A definite correlation in serologic activity was noted between Brucella antigen and Brucella abortus. Each serum sample which had a positive precipitin reaction with the extract, agglutinated the intact organism and the febrile antigen. In addition, antibodies to both Brucella antigen and to Brucella abortus cells could be removed by absorbing the antiserum with febrile antigen.

4. It appears that antibodies were not formed in response to the DNA moiety of the extract. Precipitin bands did not stain with the Feulgen

reagent, DNAase treatment of the antigen had no effect on the ring precipitin reaction and sperm DNA did not react with the antiserum.

5. Crude polysaccharide extracted from Brucella abortus G-1 produced a positive precipitin reaction with 4 serum samples. The polysaccharide component appears to be responsible, at least in part, for the antigenic specificity.

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