

A STUDY OF
FRACTIONS FROM BRUCELLA ORGANISMS

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I

INTRODUCTION

The problem of immunity to the group of diseases which has not yielded readily to man's prophylaxis is a teasing one. It had been thought by many that such diseases as tuberculosis and brucellosis were entirely dependent upon a favorable balance of the infectious agent within the host. It is not denied that such states exist but from recent developments in techniques of separation of biologically active materials there have already arisen new agents which may be used for immunization. Purified immunizing agents may result in reduction of side effects and avoidance of sensitizing doses; such agents contribute to the satisfaction of one's curiosity (or perhaps add to it) as to the nature of an agent (or agents) which so powerfully lays its imprint on the tissues of higher animals that for long periods after the imprint one resists the advances of the particular disease.

This work has not pretended to delve deeply into the chemical nature of the fractions which were separated. It has attempted to relate the solubility of proteins to the extraction of an effective immunizing agent in the native state from Brucella abortus.

II

HISTORICAL

The desirability of obtaining relatively pure and biologically active substances from the bacterial cell has been emphasized by the recent papers of several workers. Pillemer, Toll, and Badger (1947), Pillemer and Moore (1948) and Pillemer, Wittler, Burrell, and Grossberg (1948) have described the isolation and immunochemistry of homogeneous diphtheria toxoid and crystalline tetanal toxin; Lammana and Glassman (1947) have described the isolation of type B Clostridium botulinum toxin; Baker, Sommer, L.E. Foster, E. Meyer and K.F. Meyer (1947) reported the isolation of a crystalline antigen from Pasteurella pestis; and Bevilacqua and McCarter (1948) fractionated and characterized the components from tubercle culture filtrates. All of these workers have isolated substances which appear to be homogeneous and are biologically active in producing antibodies and/or immunity, in experimental animals. The methods employed for the isolation of various compounds are based on the multivariable system developed by Cohn, Strong, Hughes, Mulford, Ashworth, Melin, and Taylor (1946) and their co-workers for the fractionation of protein components of blood plasma in highly purified state.

A study of the literature on immunization against brucellosis shows that the methods of treating the cells or products used for immunization may roughly be grouped as follows: treatment of whole cells primarily by physical agents, treat-

ment of whole cells primarily by chemical agents, extraction of the cells, use of adjuvants and attenuation.

Killing cells by physical agents

1. Heat. Heat-killed bacterin was first employed by Birt and Lamb (1899) who injected heat-killed Br. melitensis in an attempt to immunize a man against live cultures of the same organism subsequently injected. The man became infected. Shaw (1907) and Eyre (1908) injected heat-killed Br. melitensis into the monkey and man respectively without obtaining significant protection. Nicolle, Burnett, and Conseil, working on man, (1923), Ascoli and Sanfilippo (1929), Zammitt and Debono (1930), on goats, and Dubois and Sollier (1938) on man, all claimed evidence of protection following the injection of heat-killed Br. melitensis. This work has not been substantiated and Huddleson (1942a) states that immunization of human beings has not been successful. A further failure of vaccination of man against brucellosis is reported by Howe, Miller, Kelly, Brookwalter, and Ellington (1947) in a report of 17 cases of brucellosis among a group of laboratory workers injected with a killed vaccine. No details as to how the vaccine was made were given.

Heat-killed Br. abortus bacterins have been used with equal lack of success by Bang (1906) in heifers, Macfadyean and Stockman (1909) in cows, Ascoli (1915) in guinea pigs, Zwick, Zeller, Krage, and Gminder (1920) and Smith and Little (1923) in pregnant and non-pregnant cows. Smith and Little felt some

protection was evidenced. Failures to immunize guinea pigs by injection of heat-killed Br. abortus cells are recorded by Ascoli (1915), Stafseth (1920), Hagan (1922b), Schroeder and Cotton (1924), Gwatkin (1931), Holth (1933), McEwen and Roberts (1936), and Huddleson (1947). Gwatkin also used phenolized heat-killed cells.

The only apparent success with heat-killed Br. abortus as far as is known, was immunization of rats reported by Holth (1911). However, the same worker found no protection for mice tested at the same time.

2. Ultraviolet light. Huddleson (1947) reported some protection to be evident in guinea pigs injected with irradiated cells and subsequently challenged with 1000 to 100,000 organisms of homologous and heterologous species.

3. Sonic treated organisms. Live, Sperling, and Stubbs (1945) disintegrated whole cultures of Br. abortus with high frequency sound waves. The cell free extract, precipitated and preserved with ethyl ether, was given in a series of 3 injections at 3 day intervals and immunized 16 of 18 guinea pigs while all but one of the control animals was infected by challenge doses ranging from 230 to 1800 Br. abortus organisms.

4. Mechanical disintegration. Huddleson (1940)(1943b) has used an ether extract of Br. suis and Br. abortus crushed in a wet crushing mill at low temperature. This produced a product which engendered some protection when used as a vaccine in guinea pigs challenged with 152 to 3,840 organisms and was important in bringing out the need for using low temperatures

when separating protoplasmic components.

Killing by chemical agents

1. Toluol. Immunization of cows by cells of Br. abortus killed with toluene was unsuccessfully attempted by Bang (1906). No other attempts with toluol-killed organisms were noted in the literature surveyed.

2. Phenol. Gwatkin (1931) used cultures of Br. abortus killed with 0.5 per cent phenol in attempting to immunize guinea pigs against challenge doses which must have been quite large as they consisted of 1.0 ml of a suspension of an entire 48 hour agar slant of Br. abortus removed in a few ml of saline. Huddleson (1947) got no protection from phenolized cultures of Br. abortus using considerably lower challenge doses.

3. Ether. Ether-killed cells were used in guinea pigs by Ascoli (1915) who found no protection. Live, Sperling and Stubbs (1945), Live (1949), and Larsen (1949) have found significant protection using cultures of Br. abortus killed with ethyl ether. Larsen has, in addition, found certain adjuvants enhance the effect. The earlier failure reported by Ascoli may have been due to the size of the challenge dose. The later, more successful, results employed challenge doses ranging from 230 to 1800 organisms. Sonic ruptured cell extract preserved with ethyl ether by Live et al in the work cited above seemed to be of about equal efficacy.

4. Formalin. Formalin-killed cells of Br. abortus have

been used, without success, by Gwatkin (1931), and McEwen and Roberts (1936) in attempts to immunize guinea pigs. On the other hand, using challenge doses of considerably less magnitude Huddleson (1947) found some protective effect existed when cells killed with not over 0.25 per cent formalin were used. Higher concentrations destroyed the immunogenic component. Foster (1948) found no organisms on post-mortem examination on one of two guinea pigs injected with Br. abortus cells killed by suspension in cold 1.0 per cent formalin at 4-6 C challenged with 10,000,000 Br. abortus. The second animal was infected but the spleen was smaller than that in control animals. Animals given formalin-killed cells and no challenge dose showed no evidence of infection or toxic reaction.

5. Phenyl mercuric nitrate-killed cells. Phenyl mercuric nitrate-killed cells were shown by Huddleson (1947) to be ineffective immunizing agents.

6. Alcohol. Br. abortus killed by cold 30 per cent alcohol failed to protect two guinea pigs from challenge doses of 10,000,000 organisms (Foster 1948).

7. Chloroform. Gwatkin (1931) found no immunizing effect from Br. abortus killed with 2.0 per cent chloroform.

8. Other methods. Scales and Huddleson (1936) used ground up spleens that were infected with Brucella and treated with either 2.0 per cent formalin or 2.0 per cent chloroform to kill the organisms. These preparations did not protect guinea pigs from infection following administration of challenge doses of

interplacental exudate treated with 2.0 per cent chloroform.

Fractionation

The use of extracts or fractions of bacterial cells was early shown to be of value in the case of the exotoxin of Corynebacterium diphtheriae by von Behring and Kitasato, as cited by Zinsser, Enders and Fothergill (1945) who successfully immunized animals against that organism by injection of culture filtrates.

Treatment of culture filtrates of Brucella cells was first employed by Holth in his previously cited (1911) work on heat-killed antigen. Both alcohol and ammonium sulfate were used to precipitate an antigenic material from Br. abortus culture filtrate. As with the heat treated cells, rats showed some evidence of immunization while mice showed none. Gwatkin (1931) (1935) utilized both heated and unheated culture filtrates preserved with 0.5 per cent phenol. In a later publication Gwatkin (1935) in unsuccessful immunization attempts in guinea pigs reported further failures to immunize using alcohol precipitated Br. abortus filtrate. This material was carbohydrate in nature, highly toxic, serologically active, but failed to protect guinea pigs from infection against rather heavy challenge doses. The filtrate residue left after precipitation was dried, toxicity and immunologic tests showing it to be less toxic but of no protective effect. Ammonium sulfate precipitation apparently produced components similar to those obtained by ethanol precipitation from precipitate and supernate. A toluol-ether and saline extract of dried and ground Br. abortus

and an alcoholic precipitate of culture filtrate from a rough strain of Br. abortus likewise gave no evidence of protective ability.

Pennell and Huddleson (1937)(1938) and Huddleson (1940) studied the effect of endo-antigen as an immunizing agent. This substance was an albuminoid fraction which produces antibodies and was toxic on intraperitoneal inoculation in guinea pigs. An S fraction linked to the albuminoid fraction and of carbohydrate nature did not produce antibodies. The nitrogen content of the endo-antigens of the various species of Brucella did not always seem to be intimately connected with the ability to precipitate specific antibodies. In immunization experiments endo-antigen failed to protect guinea pigs.

Stahl, Pennell, and Huddleson (1939) and Huddleson (1940) reported a protein nucleate fraction extracted by 3 and 5 per cent NaOH, split with sodium carbonate, and separated with chloroform, to be antigenic in 19 of 20 guinea pigs when given in 10 mg doses. The toxicity of this fraction was low but it too failed to protect guinea pigs from infection. The nucleic acid portion of protein nucleate was only feebly antigenic by itself. Precipitin studies of the fraction with smooth strain antisera of Br. abortus, Br. suis, and Br. melitensis were all positive in dilutions of from 1:1000 to 1:32,000, giving non-specific cross reactions within the group. Intermediate rough and rough strains gave no precipitin reactions. Allergic reactions occurred following inoculation in dilutions of 1:200 to 1:8000 with cross-sensitization in dilutions of 1:1000 with

rough strains.

Pop, Damboviceanu, Barber and Marinou (1938) and Damboviceanu, Barber, Pop, Marinou (1938) reported that an antigen from cells extracted in the cold with 15 per cent trichloroacetic acid, after the technique of Boivin and Mesrobianu (1934), produced antibodies in the rabbit, and was quite toxic for the mouse though less so for the guinea pig. The antigen was carbohydrate in nature. Davoli (1939) prepared a similar fraction with essentially the same technique which was nitrogen free, also toxic for mice, and produced antibodies in rabbits. This fraction precipitated homologous antiserum.

The ethyl ether extracts prepared by Live, Sperling and Stubbs (1945), Live (1949), and Larsen (1949) were briefly discussed earlier and will not be discussed further.

Miles (1939), Miles and Pirie (1939 abcde), and Patterson, Pirie and Stableforth (1947) have isolated a highly antigenic component by a combination of differential centrifugation and extraction of Er. melitensis and Er. abortus with 2.0 per cent phenol in the cold. This material produced antibodies upon injection into guinea pigs and was composed of polysaccharide, amino, and phospholipin residues. The latest publication cited found protection equivalent to that produced by Er. abortus strain 19 vaccine, to be produced upon inoculation into guinea pigs. Miles and Pirie (1939c) made the important observation that their compound was probably similar to some isolated by other workers and the superior efficacy of the antigen probably was due to the gentler treatment employed in preparing

the antigen. Indeed they were able to show a progressive loss in titer correlated with degree of hydrolysis of the antigen.

In a recent work Larsen (1949) has used ether extraction of cells previously treated with phenolized saline and separated from the liquid after 4 weeks' storage at 4 C. The cells were resuspended in 0.85 per cent saline and, with two volumes of ethyl ether added, vigorously shaken and allowed to stand 2 days at room temperature. The aqueous phase, centrifuged free of cells, was used for injection.

Serological studies on Brucella fractions have emphasized the works of Evans (1918)(1923), Feusier and Meyer (1920), and Francis (1931) showing the close relationship of the Brucella species and separating them into serological groups Br. abortus-suis and Br. melitensis. No simple separation beyond this has been made. In fact, the antigenic structure according to the work of Wilson and Miles as cited by Miles (1939) and Miles and Pirie (1939 abcd e) of the two groups is probably dependent upon a preponderance of either A (abortus-suis) substance or M (melitensis) substance. Absorption techniques can absorb the antibodies for one or the other from antiserum to produce an antiserum specific for either the A or the M antigen. Miles and Pirie (1939b) were not able to separate the antigens by fractionation. Studies on hypersensitivity and precipitin tests by Stahl, Pennell and Huddleson (1939), Huddleson (1940), Pop et al (1938) and Damboviceanu et al (1938) using previously mentioned fractions have further emphasized the difficulty of separating these species serologically. Mazzi in 1922 and Winni in 1930 as cited by

Gwatkin (1932) found immune serum to give protection against both homologous and heterologous species.

Use of living vaccine

With the work by Evans (1918)(1923) establishing the relationship of Br. abortus of bovine and porcine origin with Br. melitensis and demonstration that Br. abortus could be isolated from milk it was recognized that the earlier practice of "immunizing" cattle with living vaccine against abortion was a health hazard. The work by Giltner, Huddleson, Clark, and Schlingman (1929) on the vaccination of cattle with a strain of live Br. abortus non-infectious for guinea pigs and pregnant and non-pregnant cattle (Huddleson 1922) showed such organisms could be injected without apparent harm to cattle. Buck (1930) screened several strains of Br. abortus and found one of moderate virulence, strain 19, which did protect calves against subsequent infectious doses. This vaccine has since been widely used in cattle by many workers in this country and Huddleson (1942a) has adequately reviewed the usage. Menthel (1948) found strain 19 to produce immunity of short duration upon inoculation into 4 to 6 month gilts. McTwen (1937) has reported on another avirulent strain and the use of avirulent strains of Br. suis in emulsions of lanolin are mentioned in the following section. Huddleson (1947) found mucoid dissociants of Br. suis and Br. melitensis to be effective in protecting guinea pigs against challenge doses of 1×10^3 Br. suis and 5×10^4 or 1×10^5 Br. melitensis or Br. abortus. Following

injection of 5×10^9 to 1×10^{10} viable organisms were demonstrable 3 but not 4 weeks following vaccination. Challenge was made 4 weeks following vaccination.

Unfortunately the use of the live vaccines described is not without hazard. Crawford (1948) and Gilman (1944) cite definite cases in which human beings have been infected with strain 19 vaccine. Levine and Wilson (1949) have reported the isolation of strain 19 from the milk of 3 vaccinated cows and a hog. Persistent titers found with some frequency in vaccinated cattle further suggest possible latent infections. Deem and Cross (1945) have suggested the etiologic relationship of strain 19 to cases of abortion in cattle.

Use of adjuvants

Dubois (1938a)(1938b) found an avirulent strain of Br. suis suspended in emulsions of lanolin and petrolatum markedly reduced abortions in ovine, caprine, and bovine species and no untoward results upon vaccination of pregnant cows. Velu and Zottner (1938), Cordier (1938), and Eichorn, Mingle, and Murdock (1940) found that such emulsions were sterilized upon standing in vitro but still possessed considerable antigenicity. However Eichorn and his co-workers (1940) were able to isolate viable Brucella from the original site of inoculation in the cow up to 85 days after inoculation. Kolmer, Bondi, and Rule (1939) found that mixtures of heat-killed cells of Eberthella typhosa and Br. abortus were more efficacious in producing protective antibodies for mice upon inoculation into

rabbits than heat-killed Br. abortus cells used alone. A further interesting finding was a higher degree of protection against challenge by Br. melitensis than by Br. abortus. The phenol-killed cells and ether extracts prepared from them have been shown by Larsen (1949) to have an enhanced immunogenic effect when used with Freund's adjuvant, containing dried, heat-killed M. tuberculosis but not without them. Freund's adjuvant is a water and mineral oil emulsion. Live (1949), on the other hand, found ether-killed Br. abortus vaccine to be definitely enhanced by emulsions in lanolin and mineral oil. Alum precipitated ether-killed cells were less effective.

III

EXPERIMENTAL

Rationale

The antigenic materials to be separated were considered to be labile complex proteins possibly linked to carbohydrate and lipid constituents. Hence, the techniques were those applicable to proteins and they were based on recent techniques which have shown considerable success in isolating biologically active material.

As pointed out by the physical chemists, Cohn et al (1946), Mulford (1947), Brand and Edsall (1947), solubility of a protein may be carefully regulated by varying conditions of pH, ionic strength, temperature, and concentration of organic solvents and protein. These factors may be so chosen that the desired component has, for example, a high degree, and the undesired components have a low degree of solubility, or vice versa.

The simplest procedure and one which had not been previously attempted insofar as a search of the literature could reveal, was to use the untreated culture filtrate; that is, aqueous extract. Huddleson (1943) had found the capsular substance of Brucella to be soluble in hot water and antigenic. During the course of this work it was discovered that Cotton (1948) also had demonstrated loss of capsular material during treatment of the cell and demonstrated (1949) its antigenicity in guinea pigs.

Concentrated acetone was selected in light of the success of Baker et al. (1947) in isolating an immunogenic acetone soluble material from P. pestis. Also Horrecker (1948) had mentioned its occasional usefulness in extracting enzymes.

In the first extractions it was noted that water soluble extracts contained the enzyme urease. Hypothesizing a possible relationship in nature of the Brucella urease to that of the urease of jack bean meal, similar conditions were chosen to those applied by Sumner (1926) who used 30 per cent acetone, refrigerator temperatures, and a pH of 5.2 in crystallizing urease.

It was the intent of this work to extract antigenic material by solution in the extractant, taking advantage of several theoretical factors brought out by physical chemical studies on proteins:

1. Isoelectric point. That isoelectric point affects the solubility of large molecules is well known. In fractionation, extracts of water soluble material were made near the neutral zone (flow sheet fraction I), the pH of a plain aqueous extract being about 6.9. Extraction was also approached from the acid (fraction VII) and alkaline (fraction VI) sides of that zone. Other important considerations were the effect pH has on stability of certain compounds and on enzymes. It was considered that a much higher or lower pH might inhibit the activity of autolytic enzymes should such be present. The possibility that a given component might, in itself, be more stable in a different pH was also considered. pH was a

factor recorded in all cases but further adjustment was not considered necessary in the case of fractions I, II, and IV, the pH of about 6.9 of the medium itself being used. Fractions V-1 and V-2 were produced from a cell suspension buffered to pH 5.2.

2. Ionic strength. Low concentrations of ions have been demonstrated to have greater specificity of action for given proteins. Combined with the proper pH, sharper separation of various proteins can be obtained. The ionic strength was not varied in most of these separations but kept at that of the original extract of medium. It may be recalled that tryptose agar had 0.5 gm of sodium chloride per liter. The ionic strength based on molar solutions would be:

$$\Gamma = \sum_i C_i Z_i^2$$

Where C_i is the concentration and Z_i the valence of the i 'th species of ion in moles per liter or the ionic concentration. This is about twice the molar ionic strength (μ) as calculated by Lewis and Randall (cited by Daniels 1948). And since the salt would be .0086 molar:

$$\Gamma = (.0086 \times 1^2) - (.0086 \times 1^2) = .0172$$

$$\text{or } \Gamma/2 = \frac{.0172}{2} = .0086 \approx \mu$$

This would be the ionic strength in the medium. However, the concentration in an aqueous extract of growth from agar medium

would be considerably less. It was not measured, but should be reproducible for the given medium. Other salts were assumed to be present in negligible amounts. In the case of separation of a cell residue with subsequent reconstitution in another solvent it was assumed that the residual ionic strength from cellular debris was negligible and ionic strength was based on that of the fluid used for reconstitution.

3. Temperature. Temperature control, in this work, was designed to minimize denaturation and was practiced with all fractions. There is, generally, also an enhancement of precipitation of a given protein at its isoelectric point as the temperature is lowered and this, combined with the specific salt effect, no doubt had some effect on solubility.

4. Concentration of organic solvents. Solvents such as acetone and methanol decrease the dielectric constant of a medium and thus lead to an enhancement of interactivity between proteins and ions. A further consideration is the solvent effect. Combined with the factors mentioned above are differences in solubility in differing percentages of the solvent used. Acetone was used in high concentration for treatment of fractions II and IV and in 30 per cent concentration for treatment of fractions V-1 and V-2. Methanol was used in precipitating antigenic material from fractions I and II.

5. Concentration of protein. The concentration of a given protein affects its interaction with ions and solvent molecules. Temperature affects the interaction too. Thus

the five factors listed are mutually dependent. Furthermore, a given protein may be affected by the presence of other proteins, carbohydrates or fats. No method of control of protein concentration, beyond the quantities of the various amounts of liquids used for extraction, was practiced.

That other factors may influence solubility of proteins is recognized. The five factors listed were the only ones which were varied in a controlled manner in this investigation.

Methods

Cultivation and characteristics of Brucella species used.

All cultures used in this study were tested for species identity and conform with the current classification in Bergey's manual (Breed et al 1938). The cultures used and a partial history are included in table 1.

Table 1
Brucella Species Studied and Source

Organism	Strain no.	Source	Year	Institution
Br. abortus	19	vaccine bottle	1949	BAI ²
Br. abortus	128-A	field strain	1949	BAI
Br. abortus ¹	2132	bull	1949	BAI
Br. abortus	2308a	aborted fetus	1948	BAI
Br. abortus	2308b	cow's milk	1948	LSSS ³
Br. melitensis	1 M	goat	1949	BAI
Br. melitensis	16 M	kid	1949	BAI
Br. suis	41-8	exposure culture kept virulent by animal passage	1949	BAI
Br. suis	298	lyophilized stock culture	1948	LSSS
Br. suis	16303	field strain	1949	BAI

¹ Only CO₂ requiring strain used.

² Bureau of Animal Industry, Beltsville, Md. through the courtesy of Dr. A. E. Crawford, on potato infusion serum agar.

³ Livestock Sanitary Service Laboratories, College Park, Md. through the courtesy of Dr. Cornelia M. Cotton. Grown on tryptose agar.

Br. abortus. The variable results found here might be due to differences in the test media used.

Table 2

Number of Minutes Required by Brucella Species
to Hydrolyze Urea

Strain	Species	Time
128-A	Br. abortus	180
2132	Br. abortus	180
2308	Br. abortus	240 (very weak)
1 M	Br. melitensis	210
16 M	Br. melitensis	210
41-S	Br. suis	15
16303	Br. suis	15

All cells standardized on basis of BAI antigen at 61.3 $\pm$ 0.2 per cent transmittance using filter, wave length of 650 millimicrons on Fischer Spectrometer.

Tests for antigenic variation were carried out according to the techniques of Alessandrini and Sabatucci (1931), Ardrey and Di Aichelburg as cited by Huddleson (1943). According to Alessandrini and Sabatucci rough strains of Brucella species will agglutinate when suspensions of organisms are mixed in equal volume with 1:10,000 trypanflavine and incubated at 37 C for 6 hours. Braun and Hauge (1948) place considerable reliance on this test. No visible agglutination occurred in any of the 6 cultures used. Di Aichelburg's technique employs suspensions of organisms mixed with 1:2000 basic fuchsin in

equal volume and incubated at 37 C for 2 hours. Agglutination here also indicates a rough strain and none of the strains employed were agglutinated. Ardrey showed that intermediate strains could be detected by failure to agglutinate upon heating when water was substituted for saline in dilutions of acriflavine whereas smooth cultures agglutinated. On this basis all of the cultures employed are intermediate strains.

All of these strains were used for comparative serological studies. Only Br. abortus 2308 was used for production of immunizing antigens and challenge doses. All cultures were inoculated on tryptose agar slants (appendix I) upon receipt and kept in the refrigerator after 48 hours' growth at 37 C. Transfers were made at approximately monthly intervals.

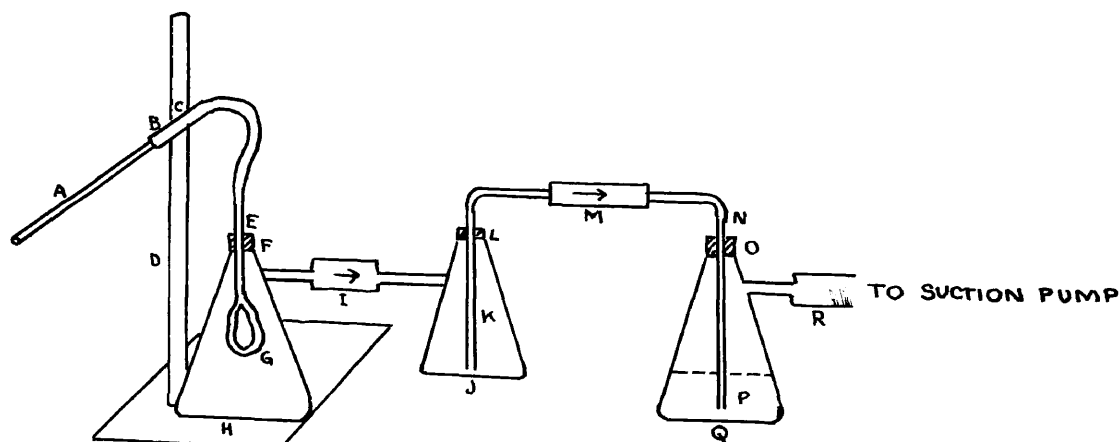
Preparation of antigens. Cells were washed from tryptose agar slants with 48 hours growth for the various tests by 0.9 per cent saline or, in the case of intermediate strain tests, by water and the resultant suspensions standardized to 61.3 $\pm$ 0.2 per cent light transmittance. This figure was selected for use of the cells in agglutination and other tests and was based on the average of several separate readings made on Bureau of Animal Industry (BAI) phenolized antigen.

Antigen for extraction was grown in Blake bottles on Difco tryptose agar reinforced with 1.5 per cent additional agar. Enriched tryptose agar (appendix I) was used in growing antigens for preparation of fractions V-1 and V-2. Seed cultures were first grown for 48 hours at 37 C allowing 1 tryptose agar slant per 6 Blake bottles. The slants were

washed off with 3 - 5 ml saline suspending the cells from each slant in 15 - 20 ml of saline. Three ml of suspension was spread over the agar surface in Blake bottles which were then incubated for 48 to 72 hours at 37 C, the growth being used for the various extractions. Antigens of live organisms used for animal inoculation and serological tests were prepared from 48 hour growth on agar slants, the cells being washed free by 0.9 per cent saline. These suspensions were diluted to 61 per cent light transmittance and used without further treatment. Collection of fractions was by means of suction flask as illustrated in figure 1.

Figure 1

Suction Apparatus for Collection of Cells
from Blake Bottles



A Glass tube for collecting cells
B Pressure tubing
C Clamp holding tubing (B) and
inserted glass tube (A)
D Ring Stand
E Glass tubing inlet to flask (H)
F One hole rubber stopper
G Two layered gauze filter
H Two liter pyrex suction flask
(for cell collection)
I Pressure tubing coupling

J One liter pyrex suction
flask (trap for back-
flow)
K Glass tube (outlet)
L One hole rubber stopper
M Pressure tubing coupling
N Glass tube
O One hole rubber stopper
P Five per cent phenol
Q Two liter pyrex flask
R Pressure tubing to
suction pump

Two traps, one (J) to avoid backflow, and the second (Q) containing 5.0 per cent phenol to trap organisms that might be pulled over were used. Five per cent phenol had the advantage of not foaming. The intake tube on the collection flask (H) was covered with a double layered gauze bag to catch large particulate matter. The outlet went through the traps to a water pump. In use all flasks were clamped to a single ring stand so that after use it was a simple matter to autoclave the entire set up.

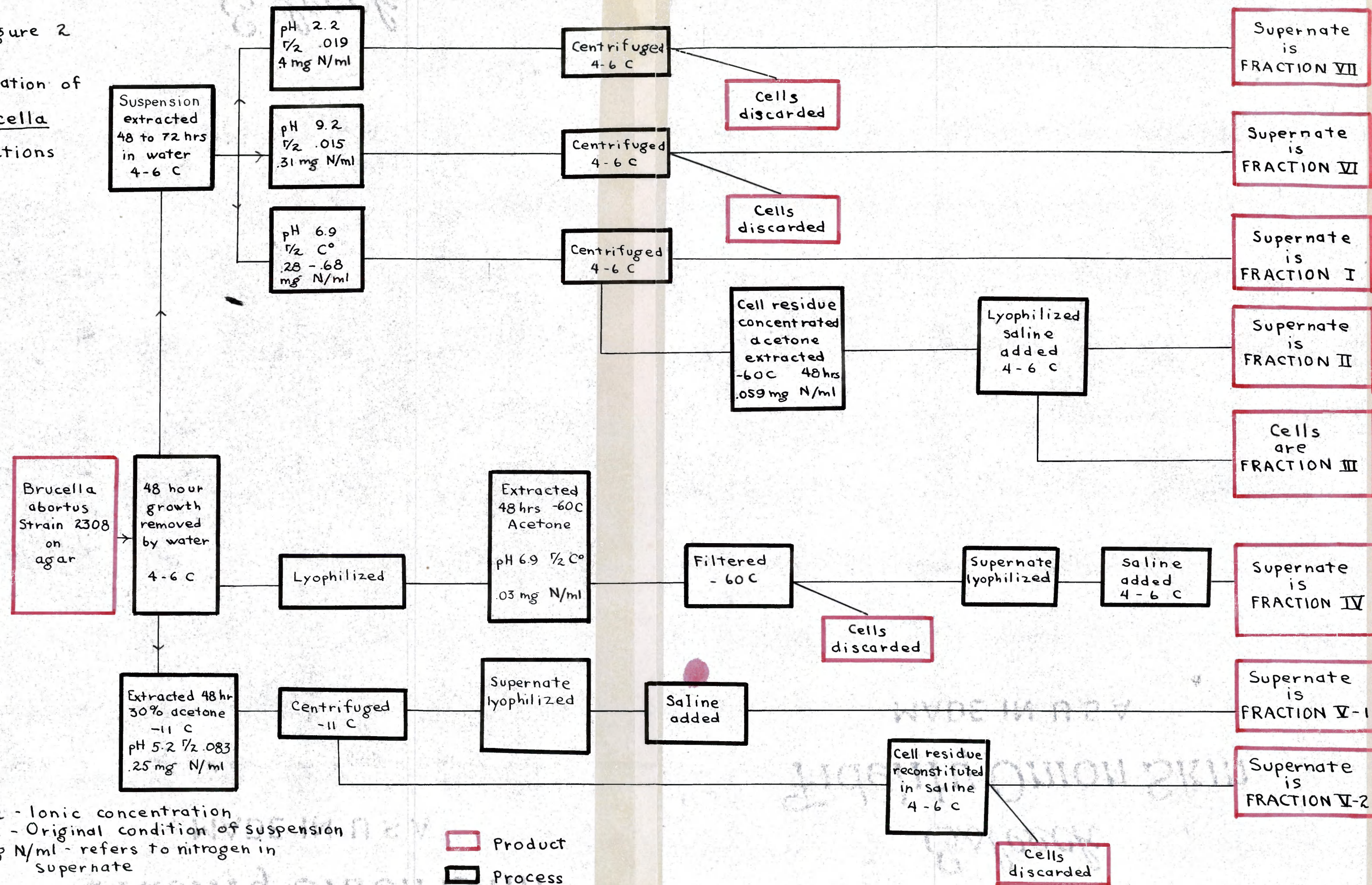
Temperature control. Temperature control during fractionation was varied. For most work between 4 - 6 C a large "walk-in" refrigerator was used. For lower temperatures a bath of alcohol or acetone and dry ice was convenient. By keeping one thermometer in the ice bath and one in the specimen large differences in temperature were avoided. Addition of small, 1.0 to 2.0 cm, pieces of dry ice maintained temperature within a degree of that which was desired. Temperatures of about -60 ± 10 C were obtained in a dry ice box. The freezing compartment of a small laboratory refrigerator happened to be maintained at -11 C. For centrifugation a box about 38 cm high by 60 cm long by 38 cm wide and having a hinged lid closing tightly over a rubber gasket was used. No laboratory infections occurred by this method as far as is known. A thermometer was placed so the bulb was at the height of the centrifuge head and only a few cm from it. About 2 kilograms of dry ice placed in the box was sufficient when broken in small pieces if the centrifuge was run at 4200 RPM to reduce

the temperature to between 0 to 5 C. Running the centrifuge 20 to 30 minutes cooled the head off and centrifuge tubes, already at the desired temperature, were then placed in the head. Addition of dry ice usually was not necessary at this time but, after another 30 minutes' run, about 500 gm more of ice was added. A 50 pound cake of dry ice usually lasted the better part of two days under these conditions. For temperatures about -10 C around 6 kg of dry ice were required at the start of centrifugation and a cake of dry ice lasted only one day.

Preparation of fractions. Explanation: The over-all plan of fractionation is expressed schematically in figure 2. Starting with 48 hours growth of Br. abortus 2308 at 37 C on agar in Blake bottles cooled to 4 - 6 C the variations in treatment of the cells are given. The conditions controlled in extraction, ionic strength ($\tau/2$), pH, temperature, solvent concentration and nitrogen content are presented. Nitrogen content being readily measured was used instead of protein concentration. The term C^0 in reference to ionic strength refers to that already present in the medium.

Designation of fractions: Roman numerals were used to indicate different fractions; arabic numerals minor variations from the main procedure. Capital letters were used to indicate different batches of a given fraction. As an example, the designation II-D-3 refers to the D batch of fraction II in which the cells of Br. suis were extracted. That is, the third variation in the major fraction. For convenience, the

Preparation of Brucella Fractions



$r_{1/2}$ - Ionic concentration
 C^0 - Original condition of suspension
 mg N/ml - refers to nitrogen in supernate

designation given the fraction was also given to groups of animals inoculated with a fraction so that group I-B guinea pigs, for example, had received the B batch of fraction I.

Fraction I. Blake bottles seeded and incubated as described in the foregoing section were placed in the refrigerator along with pipettes, flasks of sterile distilled water, and collecting flasks and cooled to 4 - 6 C. After cooling 3 to 4 hours, 10 - 15 ml of cold sterile water was added to each bottle and allowed to soak for 10 to 20 minutes. Following aseptic precautions, growth was gently loosened from the agar surface with a moistened swab, the suspension washed down with 10 ml more of cold water, and aspirated off into the collecting flask. Several uninoculated bottles previously inoculated with 3.0 ml sterile saline were treated in the same manner to make a control extract. The suspensions from 25 - 30 bottles were collected in this manner and yields varied from 500 - 600 ml. Suspensions were extracted for 24 to 48 hours as shown in figure 2, after which time the cells were separated from the liquid phase by centrifugation at 4 - 6 C in an angle head centrifuge at about 4200 RPM. The cells were resuspended in cold water, pooled in about 100 ml of water and recentrifuged, this supernate being added to the first separated. The cells were retained for further treatment (figure 2). The supernate, designated fraction I, was recentrifuged until it appeared clear to the eye, filtered in either selas or sintered glass filters at 4 - 6 C, and two 1.0 ml aliquots of filtrate cultured in respective tubes of tryptose

broth. If, after 3 days no growth was apparent in the culture tubes, injections of extract were carried out.

Variations in batches of fraction I consisted of centrifugation 1 time of fraction I-B at 15 C instead of 4 - 6 C, and use of a first application of 15 ml of cold water instead of 10 ml in removing cells from Blake bottles in Batch I-B.

A series of 4 batches of fraction I designated I-A, I-B, I-C, and I-D are reported on herein. Br. abortus 2308a was used in producing I-A, I-B and I-D. Br. abortus 2308b was used in preparation of I-C. Three of these, I-A, I-B, and I-C, were studied for antibody production and immunizing capacity in guinea pigs and as precipitins and I-C was also studied for antibody response in rabbits. The fourth, I-D, was studied in vitro only. Extracts of cells of Br. abortus 19 and Br. suis 298 designated I-D-2 and I-D-3 respectively were also made as for I-D. Cold 0.9 per cent saline was substituted for water in parallel batches; all 6 of these batches were studied for possible strain differences.

Fractions II and III. The cell residue left after separation of fraction I was cooled to 1.0 C in an acetone and dry ice bath and gradual addition of precooled acetone (0 C) made during continual stirring. Overly vigorous stirring was avoided as it is known that some proteins are susceptible to denaturation by too vigorous stirring. Temperature fall was at a rate of about 1.0 C per ml of solvent added, a total of about 60 ml acetone to the yield from 30 Blake bottles or about 2.0 ml for the yield from a single flask being added.

As far as is known freezing during extraction did not occur except in the preparation of the first fraction (II-A) in which only 50 ml per 30 Blake bottles was used. Extraction in the cold box was carried out for 48 hours as shown in figure 2. After extraction the batch was shell frozen in 100 ml centrifuge bottles. These were attached by pressure tubing to a Stokes lyophilizer. During drying the centrifuge bottles were immersed in a bath of dry ice and alcohol or acetone in which they were kept for about 12 to 15 hours or until the acetone vapor cleared the vacuum pump, at a temperature of -75 to -78 C. It was observed that the pressure of the lyophile apparatus first dropped under 500 microns but then rose to 1 mm or above as the acetone, raising the vapor pressure, entered the vacuum pump. As the vapor was pushed out of the pump the pressure fell and once it was again below 500 microns and remained below the alcohol and dry ice bath was considered no longer necessary.

After drying, the cell residue from 10 Blake bottles was a yellowish to brown powder weighing about 0.5 grams. To this powder 50 ml of 0.9 per cent saline at 4 - 6 C was added and the cells extracted overnight at 4 - 6 C. On the following day, cell residues were separated by centrifugation from the liquid phase and washed by a second centrifugation with about 30 ml cold saline. The cell residue was designated fraction III. The liquid phase designated fraction II was centrifuged 1 or 2 more times until it appeared clear to the eye and filtered through a selen or sintered glass filter. Two 1.0 ml

aliquots of the fraction were cultured in tryptose broth and incubated for sterility controls at 37 C three days before inoculation of guinea pigs was carried out. The broths remained sterile for the 30 day total incubation period used.

In all, 4 batches, II-A, II-B, II-C and II-D were made. II-A, II-B and II-D were prepared with Br. abortus 2308a and II-C with Br. abortus 2308b. II-A, II-B and II-C were studied for antibody production and immunizing ability in guinea pigs and II-C was also studied for antibody production in rabbits. All 4 were studied as precipitins and II-D includes 3 sub-fractions, II-D-1, II-D-2, II-D-3 from Br. abortus 2308a, Br. abortus 19 and Br. suis 298 respectively. These were studied for serological strain differences by precipitin and absorption tests.

Two batches of fraction III, III-A and III-C, are reported on. III-A was reconstituted in saline and standardized to 95 per cent light transmittance, 1.0 ml of the suspension being injected in each of 10 guinea pigs. Batch III-C was diluted with about an equal volume of saline and 0.1 ml of this suspension given intraperitoneally in each of 20 guinea pigs. III-B was not studied.

Fraction IV. The cell suspension for fraction IV was prepared as that for fraction I using one half of each suspension for fraction IV, the other half for fractions V-1 and V-2. After harvest the cell suspension was shell frozen in 100 to 500 ml pyrex bottles containing from 20 - 100 ml of cell suspension per bottle and the bottles attached to

the lyophile apparatus by pressure tubing. After the Stokes gauge remained below 500 microns, the dry ice bath was removed and dessication was continued about 20 hours more. The pressure was usually 50 - 100 microns during most of the run. Following dessication, acetone, precooled to -60 to -70 C was added to the yellowish residue. About 10 ml of acetone was added per 0.1 gm of dried cells. Extraction was carried out in a dry ice box at -60 to -50 C for a period of 48 hours under the conditions shown in figure 2, after which time the extract was filtered through a thoroughly dried selas or sintered glass filter in the dry ice box. The filtrate was designated fraction IV and the cells were discarded. The acetone suspension was shell frozen, the addition of 20 ml of cold saline being necessary to permit freezing, and bottles were left immersed in dry ice and alcohol or acetone at -75 to -78 C until the Stokes gauge showed a continuous pressure of less than 500 microns. The large volume of acetone presented a problem as the vacuum pump remained at elevated pressures for quite some time. If removed from the bath before pressure was continually low the cell suspension melted. A whitish powder remained after drying. This was reconstituted in cold saline and filtered through a selas or sintered glass filter at 4 - 6 C. Two 1.0 ml aliquots were cultured in tryptose broth for sterility. Three batches, IV-A, IV-B and IV-C were made; IV-A and IV-B using Br. abortus 2308a were used in precipitin tests, IV-C using Br. abortus 2308b was also used for guinea pig and rabbit inoculation.

Fractions V-1 and V-2. The 250 ml of cell suspension left over from preparation of fraction IV was cooled further to 1.0 C in a dry ice and acetone bath and 10 ml of acetate buffer added (appendix IV). The buffer was made from 9.0 ml 1 N sodium acetate and 1.0 ml 1 N acetic acid. Diluted to 250 ml in water alone the buffer gave a pH of 5.2 on a Beckman pH meter. No pH reading was made on the extract itself. With constant gentle stirring and gradual cooling 110 ml acetone was added, dropping the temperature 1.0 C for each 10 ml acetone added. The buffered acetone and water suspension was extracted as shown in figure 2, in the freezing compartment of an ordinary refrigerator at a temperature of -11 C for 48 hours. At the end of this period the liquid phase was centrifuged free of cells at -10 to -12 C in the cold box. Centrifugation was carried out 2 times on the liquid, pooling all cells separated. Unsterilized plastic tubes were used in place of glass ones to avoid breakage. The plastic being somewhat soluble in acetone it was desirable not to expose the tube to the acetone any longer than was necessary. The supernatant material, designated fraction V-1, was shell frozen and the acetone and water in it removed by lyophilization for 36 hours, the containing bottles being kept immersed in a dry ice bath at less than -30 C until pressure remained below 500 microns. The dried residue was a yellowish coherent mass which was readily soluble in cold water. Fraction V-2 was prepared by adding 80 ml of water at 4 - 6 C to the cell residue, centrifuging at 4 - 6 C, washing a second time with

60 ml cold water, and combining the two 60 ml aliquots. Both fractions V-1 and V-2 were filtered through selas or sintered glass filters and two 1.0 ml aliquots of each were cultured in tryptose broth. When no growth was apparent after 3 days, inoculations were made.

Three batches, V-1-A, V-1-B, V-1-C, and V-2-A, V-2-B, V-2-C, of each fraction were made. The batches were all studied for ability to precipitate antiserum and V-1-B, V-1-C and V-2-B, V-2-C were studied for immunizing and antibody producing ability in guinea pigs. V-1-C and V-2-C were also used for antibody production in rabbits.

Fraction VI. Fraction VI was prepared by the substitution of dilute alkaline buffer for water in the removal of growth from Blake bottles. The buffer (appendix V) gave a final pH of 9.0 in the extract. The original buffer diluted in water had a pH of 9.2. Ten ml of buffer were added to each Blake bottle pre-cooled as before, and allowed to soak 10 to 20 minutes before gently swabbing the suspension loose and washing down the surface of the agar with an additional 10 ml of buffer. Collection was made in a suction flask as previously described, keeping all materials cooled to 4 - 6 C. The buffered cell suspension was allowed to extract in the refrigerator under the conditions shown in figure 2, for 72 hours and the cell suspension was separated into paste and supernate by centrifugation at 4 - 6 C. The cells were discarded, the liquid being centrifuged once more and filtered through a selas or sintered glass filter. Some difficulty in filtration of

this and fraction VII was experienced it being necessary to filter the alkaline extract 3 times to achieve sterility in the second batch. Two 1.0 ml aliquots were cultured in tryptose broth and when, after 3 days incubation at 37 C no growth was apparent inoculations were made.

Two batches, VI-A and VI-B, were made; the former with Br. abortus 2308a, the latter with Br. abortus 2308b. Both were tested for immunizing capacity, ability to elicit antibodies in guinea pigs, and ability to precipitate immune serum. VI-B was also tested for antibody production in rabbits.

Fraction VII: Fraction VII was prepared by addition of cold acid buffer (appendix V) to Blake bottles with 48 hours growth at 37 C of Br. abortus, in place of the cold water as used in preparation of fraction I. The pH was 2.2, as determined on diluted buffer, not the extract. Following removal of cell growth the suspension was refrigerated at 4 - 6 C for 72 hours and centrifuged to separate the extract from cells. The extract was centrifuged once more and filtered through a selas or sintered glass filter. In both batches made it was necessary to filter the acid extract 4 times before two 1.0 ml aliquots cultured in tryptose broth showed no growth after 3 days incubation at 37 C. When no growth was apparent after 3 days a series of inoculations was made. Two fractions, VII-A and VII-B were made. Only fraction VII-B was used for animal inoculations.

Methanol precipitation. To 40 ml of cold fraction I or fraction II was added 1.0 ml 4 M sodium acetate and 1 ml 10 M

acetic acid. The resulting solution had a pH of 4.7 on the Beckman pH meter and an ionic concentration of .004 (appendix V). To the buffered filtrate was gradually added 7.5 ml of cold (00) methanol while the temperature was lowered to -17 C. A precipitate developed, the material was centrifuged at -15 C and 4200 RPM for 30 minutes and the supernate, still cloudy, decanted. A light precipitate left on the bottom of the tube was readily soluble in 10 ml of cold water. This material was precipitinogenic.

Criteria for sterility of fractions. As mentioned, two 1.0 ml aliquots of each fraction were incubated in tryptose broth at 37 C for at least 3 days before animals were inoculated except in the case of the first fraction, I-A, in which it was shown that the fraction was not sterile by control animals' infections. The broths were all kept for a minimum of 30 days before being discarded. In no case was any growth noticeable. About half of the guinea pigs inoculated with the given fractions also served as sterility controls receiving no challenge dose. In all but the one case mentioned (group I-A) these animals were sterile.

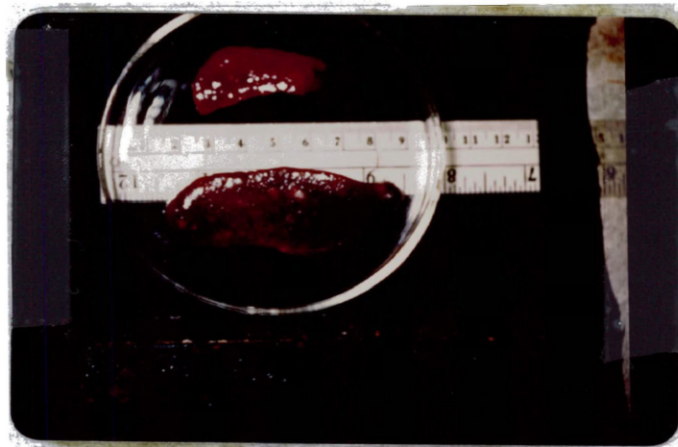
Care of animals. Guinea pigs and rabbits were fed a diet of Purina pellets supplemented with frequent feedings of kale or lettuce and adequate water. Rabbits were kept individually or paired in open cages while guinea pigs were placed in cages by groups. Isolation among the guinea pigs was imperfect. One epidemic of streptococcal infection and an extended one of enteritis occurred in the laboratory pool

of normal animals during the experiments. Not many of the animals used were involved in the streptococcal outbreak but practically all animals receiving fractions V-1-A, V-1-B, V-2-A, V-2-B and the infection controls for these groups and I-C, II-C and VI-A were involved in the enteritis outbreak. The outbreak was controlled by feeding sulfa guanidine and of the enteritis animals given post mortem examination no macroscopic pathology of the spleen was apparent. Two of 6 had small patchy abscesses on the liver. Sulfaguanidine was withheld from the animals at least 1 week prior to starting an experiment except in the infection controls for I-C and II-C which by accident were receiving sulfaguanidine up to the day they received the challenge dose.

Inoculation of rabbits. Rabbits used for the production of antiserum weighed from 5 to 10 pounds. Normal titers were taken by bleeding from the marginal ear vein before any material was inoculated and inoculations then made directly in the ear vein. At the intervals indicated on the various protocols given under "Results" trial bleedings were made. If a titer of desired strength was obtained the animal was bled out from the heart aseptically. Serum was separated after the blood had stood 2 to 4 hours at 37 C and had been refrigerated overnight. Cellular debris was removed by centrifugation and the serum aspirated off and stored in small rubber capped serum bottles. Merthiolate in final concentration of 1:10,000 was added when preservative was used.

Inoculation and post mortem examination of guinea pigs.

Plate 1



Brucella infected spleens

Guinea pigs weighed from 300 to 700 gm when inoculated. The weight variation within a group usually was $\pm$ 100 gm. Titers were obtained on all guinea pigs before inoculating and at intervals indicated under "results" following the inoculation of the various fractions. All inoculations were made by the intraperitoneal route. It was found convenient to place one animal in an ether anesthesia jar while a previously anesthetized animal was being worked on. Titers were taken just prior to each inoculation of a given fraction or challenge dose. Protocols I and II include inoculation schedules. Surviving guinea pigs were titered and killed by chloroform or ether 4 to 5 weeks following the challenge dose. One blood specimen was taken, in groups I-C, II-C, IV-B, V-1-C, V-2-C, VI-B and VII-B, two weeks after challenging in order to follow the titers more closely. Those dying by natural cause are so noted in the tables.

Post mortem examinations were done on nearly all animals recorded in these experiments with exceptions as noted in which decomposition was too advanced to make such examination feasible. Organs examined included spleen, liver, heart, lung, kidney and reproductive organs. Occasionally reference was made to abscesses in other parts of the body as swollen lymph glands, inflamed adrenals, and arthritic swellings. According to the classical description of Fabry (1912) the spleen characteristically is enlarged and roughened and may have numerous tiny abscesses over the surface (plate I). The liver is less frequently involved but also may be abscessed. The heart oc-

Protocol I

Schedule of Inoculations¹ of Various Fractions
in Guinea Pigs

Fraction inoculated	Number of animals inoculated	Ml of fraction inoculated	Days between injections of fractions		Days after injection of fraction to challenge	No. of days from challenge to titer	Plate count of challenge dose
			1st	2nd	2nd-3rd challenge		
I	10	5.0	-	-	30	30	8,200
I	20	5.0	-	-	30	14 and 30	100,000
I	20	3.0	2.0	2.0	10	14 and 30	700
II	10	2.0	-	-	30	30	8,200
II	20	2.0	-	-	30	30	100,000
II	20	2.0	2.0	2.0	10	14 and 30	700
III	10	1.0	-	-	30	30	8,200
III	10	0.1	-	-	none given	not chall.	not chall.
IV	20	1.0	1.0	1.0	9	14 and 30	10,000
V-1	20	1.0	-	-	30	30	7,600
V-1	20	1.0	1.0	1.0	10	30	14,000
V-2	20	1.0	-	-	30	30	7,600
V-2	20	1.0	1.0	1.0	10	30	14,000
VI	20	5.0	-	-	30	30	7,600
VI	20	2.0	2.0	2.0	9	30	10,000
VII	20	2.0	2.0	2.0	9	30	10,000

¹Animals were titered prior to each inoculation of fraction
²About half of animals inoculated with each fraction left unchallenged to serve as sterility control

asionally may have abscesses, and the lung shows congestion and consolidation is not infrequent in about 60 per cent of the cases. The kidney often has minute abscesses beneath the capsule and occasionally the adrenals are involved. The reproductive organs of the female do not often show macroscopic pathology though occasionally the uterus may show an abscess. Nevertheless organisms are often recovered. In the male superficial blood vessels on the testes are frequently congested, the epididymis often has a cream colored pus. Occasionally the seminal vesicles are involved. The later descriptions of Hagan (1922) and Huddleson (1943a) agree well with those of Fabyan and are in part based on lower challenge doses.

Cultures were routinely made of the spleen, liver, heart's blood, lung, kidney, and reproductive organs. Tissues were sterilized in most cases by searing the surface. The spleen was usually scraped with a sterile spatula, the pulp recovered being smeared on a tryptose agar slant and portions suspended in tryptose broth. Whole spleens removed aseptically were sometimes cultured. Small bits of tissue and blood were recovered by use of a stiff wire loop from the liver, kidney and testicles, and the lung was most conveniently cultured using a sharp sterile scalpel blade to excise small pieces of tissue. An effort was made to recover tissues from areas showing pathology though as Fabyan (1912) points out it is frequently impossible to see the lesions of brucellosis until microscopic sections are made. No histological studies were made in this work. It was found most convenient to culture

blood at the time the final bleeding was taken, placing a few drops of blood over the surface of an agar slant and a few drops into broth. A tryptose agar slant and tryptose broth was inoculated from each of the above mentioned tissues. Occasionally cultures were made from other sites as indicated in the protocols. Cultures were examined after 2 or 3 days and thereafter weekly for 1 month. No culture was discarded as negative under 30 days. Suspicious colonies were isolated where necessary and grown on tryptose agar slants. The homogeneous cell suspensions were examined microscopically with Gram's stain to insure presence of typical gram negative rods and absence of easily recognizable contaminants. Suspicious organisms were screened by slide agglutination using a drop of 0.9 per cent saline with a drop of 1:100 dilution of a homologous rabbit antiserum titring 1:20, 240 before dilution. They were finally tested for urease activity. If a culture from one organ, usually the spleen, proved positive from this evidence all suspicious cultures from other organs were considered positive. Very rarely was a suspicious colony encountered which was negative on these tests. When an organism was found to give a positive reaction with one of the tests, the other reactions were always positive. Gram stains were quite typical.

Infection controls. Five different groups of control animals were used. Where possible these animals were of the same lot ordered for tests. They were kept in separate cages under similar conditions and heart bleedings were obtained

prior to the time the challenge dose was made to insure absence of Brucella agglutinins, two weeks thereafter, and at the time of post mortem examinations 4 to 5 weeks after the challenge dose was given.

Protocol II
Infection Controls

Series	No. animals	Controlled groups inoculated challenged simultaneously
1	3	I-A, II-A, III-A
no controls		I-B, II-B
2	10	I-C, II-C, III-C
3	5	V-1-B, V-2-B, VI-A
4	5	V-1-C, V-2-C
5	5	IV-B, VI-B, VII-B

Estimate of Challenge Dose. Numbers of organisms injected were estimated on the basis of plate counts on tryptose agar plates. Preliminary studies were made on 48 hour growth of Br. abortus 2308a cultured at 37 C. Suspensions washed from agar slants in 0.9 per cent saline were standardized to 90 per cent $\pm$ 1.0 light transmittance using a 650 millimicron filter. Duplicate dilutions of 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} and 10^{-10} were plated and agar cooled to 43 C poured in the plates. The agar and dilution were mixed by rotating the plates 10 times clockwise and 10 times counter-clockwise. These were incubated 5 days at 37 C and showed the following counts: (table 5).

Table 3

Plate Counts of Suspensions of Br. abortus 2308
Grown 5 Days at 37 C and
Standardized to 90 per cent Light Transmittance

Dilution	Plate 1	Plate 2	Average	Plate count
10^{-10}	6	7	6.5	6.5×10^{10}
10^{-9}	75	79	77	7.7×10^{10}
10^{-8}	TNTC*	TNTC	--	--

*Too numerous to count.

On this basis a 10^{-7} dilution should yield approximately 7500 organisms and such dilutions were used until the plate count on one series of experiments gave a much lower count of 700 organisms. Then the dilution was dropped to 10^{-6} . Plate counts obtained are included in protocol I.

Agglutination tests. Agglutination tests for comparative serology were carried out by making twofold serial dilutions of serum ranging from 1:20 through 1:163,840 in 0.9 per cent saline. To 0.5 ml of diluted serum in each tube was added 0.5 ml of the desired antigen. Titers were expressed as final dilutions. Agglutinations on guinea pig sera were carried out using 0.01, 0.02, 0.04 and 0.08 ml of serum in respective tubes to which were added 2.0 ml of BAI tube antigen giving final dilutions of 1:200, 1:100, 1:50 and 1:25 respectively. Dilutions of 1:2000, 1:1000, 1:500 and 1:250 were made following pipetting 0.1 ml of serum into 0.9 ml saline. From this 1:10 dilution were delivered 0.01, 0.02, 0.04 and

0.08 ml to respective tubes. Again 2.0 ml of antigen was added. Dilutions higher than 1:2000 were rarely carried out for guinea pig serum, it being assumed that a titer of 1:2000 was quite significant. The phenolized antigen and serum mixture was incubated for 48 hours. Where living antigen was employed, 2 to 4 hours incubation at 37 C was followed by refrigeration for 48 hours. Readings on agglutinations were made considering a four-plus reaction as one in which agglutination and clearing was complete, a three-plus one in which agglutination and clearing was between 75 and 100 per cent complete, a two-plus one in which agglutination and clearing was between 50 and 75 per cent complete, a one-plus one in which agglutination and clearing was between 25 and 50 per cent complete, and a plus-minus one which showed less than 25 per cent agglutination and clearing. Control tubes containing the antigen under study were included when the tests were run.

Precipitin tests: Two different techniques were used; in the first, precipitin tests were carried out on twofold serial dilutions of fractions involved. Serum was added undiluted, or diluted 1:10 times. Proportions of serum and antigen varied. Controls consisting of solutions of extract alone and serum alone were run with each test. Titers were expressed as the last tube showing clouding or distinct precipitate. Both types of precipitin tests were incubated 2 to 4 hours at 37 C and 48 hours at 4 - 6 C.

The second technique involved quantitative precipitin

tests. Serum used for these tests was centrifuged at 4 - 6 C just prior to use. Total nitrogen was determined separately on fractions and sera and after incubation of the mixtures on the supernatant liquid following centrifugation at 4 - 6 C. In determining nitrogen 1.0 to 2.0 ml aliquots were used. Some attempt to demonstrate differences between species and strains was made but it must be borne in mind that zones of optimum reaction were not determined. Comparison of the precipitating efficacy of the various fractions was also used. Details of the method of determining nitrogen are in appendix IV. Once nitrogen values were obtained the following formula was used to calculate total nitrogen in serum-antigen mixtures:

mg N per ml of mixture =

$$\frac{\text{total mg N in serum used} - \text{total mg N in antigen used}}{\text{total volume of serum-antigen mixture}}$$

From this value was subtracted the actual amount of nitrogen found in the supernate of reacted mixtures of antigen and antiserum following centrifugation in the cold. The remainder was a measure of antibody nitrogen and antigen removed from the mixture. Since the antigens were not highly purified, precise measure of antibody nitrogen could not be made by this method. In cases in which precipitates were measured, precipitate nitrogen should closely equal the difference between calculated total antigen-antibody nitrogen and measured supernate nitrogen after precipitin reaction. The techniques were based on those presented by Kabat and Mayer (1948).

Chemical studies. Studies on the chemical nature of the various fractions were not done in great detail. Evidence of protein nature rested on the biuret test for peptide linkages, the ninhydrin test for alpha amino radicals (appendix IV) and antigenicity upon inoculation in experimental animals. Carbohydrate was tested for by the Molisch test and the presence of reducing sugars before and after hydrolysis were tested for by use of Benedict's and Barfoed's reagents (appendix IV). It was felt that the present problem in itself was sufficiently extensive that the quantitative aspects would be better reserved for a special inquiry. Tests for fat were not carried out. Chromatographic techniques based on those of Martin (1947) were used to point up some differences in extracts. n-Butanol was saturated with water by shaking in a separatory funnel retaining the butanol layer. The tank for the test, a large air tight wooden box with paraffin coating, was allowed to become thoroughly saturated by placing a tray of water saturated n-butanol on the bottom. A tall glass cylinder 30 cm wide by 56 cm tall was also used in which case water saturated butanol was poured directly on the bottom. In either case vaseline or plasticene was used about the rim to seal the lids. The tank was then kept closed for at least 24 hours prior to use. 0.01 ml of extract, unhydrolyzed, was placed about an inch and a half from the bottom edge of a large dry sheet of Whatman #2 filter paper cut to the size desired. Several extracts could be run simultaneously by placing respective drops 4 to 5 cm apart on a line drawn parallel to the bottom edge of the paper.

The paper was hung so that the vertical edge was perpendicular to the surface of the butanol in the bottom of the tank and so that the bottom edge was evenly immersed to a depth of 1.0 cm in the solvent. A drop of ammonium hydroxide was allowed to run down the side of the tank, the chamber was sealed. The solvent was allowed to advance upward on the paper for 48 hours. At the end of this period the paper was removed, dried rapidly with a fan and 0.1 per cent ninhydrin in n-butanol sprayed as evenly as possible over the paper. This also was dried quickly in front of a fan and color of components in fractions was well developed within 24 hours without heating. A convenient device for spraying was found in the sprayer used with the commercial product Windex. A spraying device made of plastic is of course not suitable. No hydrolysates of fractions were run; hence no attempt was made to establish amino acid compositions.

Changes in morphology. Examination for capsules was made using the technique of Cotton (1948). A loop of a small suspension of cells in 0.9 per cent saline was placed on one end of a clean slide and a thin film made as for a blood film. This was rapidly dried under a hair drier and a loop of india ink, placed where the previous loopful of cells had been, was spread over the thin film of organisms. This also was rapidly dried and the slide fixed in absolute methanol for a minute. Cotton's technique calls for counter staining with saturated crystal violet 5 minutes but it was found equally convenient to counterstain in carbol fuchsin 5 minutes. Under the optical microscope the capsule was shown as a distinct clear or

slightly tinted area evenly outlined by the ink background while the cell proper was generally centrally located and stained a deep blue or red. Smears were made from cells before any extraction and following extraction of I-C, II-C, IV-C, C-1-C and V-2-C, VI-B and VII-B respectively.

Electron micrographs were made through the courtesy of Maj. R. E. Reagan of the Live Stock Laboratories. Specimens suspended in saline were heat-killed at 80 C for 10 minutes and observations made on cell residues left after extraction of fractions I-B, II-B, V-1-A and V-2-A and VI-A. Photographs were taken at magnifications indicated in "results".

IV

RESULTS

Immunization of Guinea Pigs

Of the fractions studied, numbers I, II and VI gave most promise of protection against infection. Fractions V-1 and V-2 appeared to give some protection. Fractions VII and IV failed to give any evidence of protection. The cell residue left after extraction of fractions I and II contained living cells. The fractions are considered individually below.

Fraction I. The inoculation of a single 5.0 ml dose of fraction I-B in 20 guinea pigs failed to confer protection in 10 guinea pigs challenged 4 weeks later with 100,000 organisms (table 4). Agglutinin titers were shown in all of the infected animals. Recovery of Brucella correlated with a mean spleen weight of 2.0 gms in the infected animals. Of the 10 unchallenged animals receiving the fraction, nearly all had some thickening or nodulation of the spleen, and the spleens had a mean weight of 1.24 gm though final titers were negative in 1:25 dilution and no cultures were recovered in any case. These animals weighing 400 to 600 gms at post mortem were not large enough to have normal spleens weighing over 0.6 to 0.9 gm. A spleen of 1.0 gm is unusual in a completely normal guinea pig even in weights over 1000 gms.

Infection controls were omitted, but comparison of the challenged animals of this experiment with infection controls used in other experiments and with results of Hagan (1922a)

Table 4

Macroscopic Pathology and Cultural Results
in Group I-B Guinea Pigs

guinea pig number	sex	chal- lenged	Macroscopic Pathology							week of death
			spleen wt.gms.	pathology	liver	heart	lung	kidney	reproductive organs	
101	M	no	1.1	sl th nod	few abs	n	sl cong	n	n	PM
102	M	no	1.1	sl th nod	n	n	sl cong	n	n	PM
103	F	no	1.7	sl th nod	n	n	sl cong	n	5 wks preg	PM
104	M	no	1.0	sl nod	n	n	n	n	n	PM
105	F	no	2.0	sl nod	n	n	n	n	3 wks preg	PM
106	M	no	1.2	n	n	n	sl cong	n	n	PM
107	M	no	0.9	sl th nod	few abs	n	cong	n	cong testes	PM
108	M	no	1.1	sl th	few abs	n	mkd cong	n	n	PM
109	F	no	1.4	sl th nod	n	n	n	n	4 wks preg	PM
110	F	no	0.9	n	n	n	mkd cong	n	6 wks preg	PM
121	M	yes	2.4	nod th abs*	n	n	cong atro- phy	n	n	PM
122	M	yes	1.5	sl nod th*	sl enl abs	n	mod cong	n	sl cong testes	PM
123	F	yes	1.7	nod th*	n*	n	cong	n	3 wks preg*	PM
124	F	yes	1.4	sl th nod*	few abs	n	sl cong	n	live birth (2)	PM
125	M	yes	2.0	mod th sl nod*	n	n*	n	n	n	PM
126	F	yes	1.5	sl th nod*	few abs	n	sl cong*	n*	early preg*	PM
127	F	yes	0.8	n*	n	n	cong*	n	early preg	PM
128	M	yes	3.8	mkd th nod*	abs*	n	sl cong*	n*	n	PM
129	F	yes	2.3	nod th*	abs*	n	sl cong*	n	early preg*	PM
130	M	yes	2.6	nod th*	sl enl abs*	n	mod cong	n	n	PM

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

Key:

sl - slight	th - thickened	nod - nodular
n - normal	abs - abscess	cong - congested
preg - pregnant	mod - moderate	mkd - marked

(1922b) with similar strength challenge doses suggested little if any modification of the infection by the fraction. The fraction itself seemed to have contained some toxic material, as shown by the slightly enlarged spleens in unchallenged animals.

Three inoculations of fraction I-C at 10 day intervals followed by a challenge dose of 700 organisms following the last injection of fraction by 10 days resulted in prevention or modification of the infection in 9 of 10 guinea pigs as shown in table 5. Brucella were recovered from the spleen of guinea pig number 436. The mean spleen weight of the challenged group was 1.37 gm as opposed to 1.20 gm for the infection controls (table 6) and 1.07 gm for the unchallenged animals of the same group. The mean spleen weight of infected challenged animals was 1.6 gm. Inasmuch as the infection controls had been receiving sulfaguanidine up to the day of receiving the challenge dose, the lower spleen weight was probably not significant. The titers among the group receiving the fraction and challenge supported the negative cultures very well except in the case of guinea pig number 434 which showed a definite increase in titer after having no detectable agglutinins 10 and 20 days prior to the final titering.

The marked difference in the protective power of the two fractions may be explained in part by the absence of precipitating power on the part of fraction I-B, and possibly a lack of antibody response in guinea pigs. Of further interest is the accompanying loss of urease activity.

Table 5

Macroscopic Pathology and Cultural Results
in Group I-C Guinea Pigs

guinea pig number	sex	chal- lenged	Macroscopic Pathology							week of death
			spleen wt. gms.	pathology	liver	heart	lung	kidney	reproductive organs	
431	F	yes	1.2	n	n	n	n	n	preg no abor- tions obsvd.	PM
432	F	yes	2.6	sl nod	n	n	n	n	live birth	PM
433	F	yes	1.0	n	n	n	n	n	live birth	PM
434	M	yes	1.4	sl th	n	n	n	n	n	PM
435	F	yes	1.4	n	n	n	n	n	full term preg	PM
436	M	yes	1.6	sl th nod*	abs	n	sl cong	n	sl cong testes	PM
437	M	yes	0.8	n	n	n	n	n	sl cong testes	PM
438	F	yes	1.2	sl th	l sm abs	n	n	n	full term preg	PM
439	F	yes	1.5	sl th	n	n	n	n	live births	PM
440	M	yes	1.0	n	n	n	n	n	n	PM
441	F	no	0.9	sl th nod	n	n	n	n	preg 6-7 wks	PM
442	F	no	1.0	sl nod	n	n	n	n	preg 4-5 wks	PM
443	M	no	1.0	sl nod	n	n	sl cong	n	n	PM
444	F	no	1.2	n	n	n	sl cong	n	preg 5-6 wks	PM
445	F	no	0.9	sl nod	n	n	n	n	preg 5-6 wks	PM
446	F	no	1.1	sl th nod	n	n	n	n	preg 5-6 wks	PM
447	M	no	1.2	sl th nod	n	n	n	n	n	PM
448	M	no	0.9	sl th nod	n	n	n	n	n	PM
449	F	no	1.4	sl th	n	n	n	n	preg 5-6 wks	PM
450	M	no	0.3	n	n	n	sl nod	n	n	PM

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

Key:

sl - slight	th - thickened	nod - nodular
abs - abscess	preg - pregnant	n - normal
cong - congested		sm - small

Table 6

Cultural and Pathological Results in Guinea Pigs Inoculated with
Challenge Used for Groups I-C and II-C

guinea pig number	sex	Macroscopic Pathology					week of death		
		wt. gms.	spleen pathology	liver	heart	lung			
B	M	0.9	sl th nod*	abs	n	sl cong*	n	mod cong testes	PM
F	F	0.9	sl th nod*	n	n	mod cong	n	preg 3-4 wks	PM
G	M	0.9	sl th nod	sl enl	n	mod cong	n	sl cong testes	PM
H	M	1.5	mod th nod*	num abs enl*	n	mod cong	n*	epididymitis*	PM
I	M	0.7	sl th nod*	n*	n	sl cong	n	n	PM
J	M	2.6	mod th nod*	num abs enl*	n	sl cong*	n*	mod cong*	PM
K	M	1.1	sl th nod*	abs*	n	sl cong*	n	mod cong	PM
L	M	0.9	sl th nod*	n	n	n	n	n	PM
M	M	1.1	sl th nod*	hem area*	n	n	n	n	PM
N	M	1.4	sl th nod*	few abs*	n	n	n	n	PM

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

Key:

sl - slight	th - thickened	nod - nodular
mod - moderate	abs - abscess	n - normal
num - numerous	enl - enlarged	hem - hemorrhagic
preg - pregnant		

Fraction II. On the basis of cultures, no infection existed in the 5 challenged group II-A animals at the time of post mortem examination. Table 7 summarizes the pathological and cultural results on control and challenged animals receiving fraction II-A. As in animals receiving fraction I, some thickening and nodulation of the spleen existed but the mean weight of the spleens of challenged animals receiving fraction II-A was only 0.74 gm. This compared with mean weights of 1.08 of the spleens of the 5 unchallenged animals receiving fraction II-A and 0.8 in infection control animals (table 8). The unchallenged animals receiving II-A (sterility controls) were sacrificed 3 weeks following inoculation while the infection controls were sacrificed 4 weeks following inoculation of the challenge dose or 7 weeks after inoculation of the fraction. These controls had a mean titer of over 1:800 and were infected. (tables 7 and 8).

All 10 guinea pigs inoculated with 2.0 ml of fraction II-B and subsequently challenged with 100,000 Br. abortus 2308 were infected. Ten animals receiving the fraction only showed no evidence of Brucella as shown in table 9. However, some thickening and nodulation of the spleens was evident. The mean weight of the spleen was 1.01 gm in sterility controls and 3.55 gm in animals inoculated with the fraction and subsequently challenged. This size was not attained in most of the infection controls used in these experiments.

Agglutinin titers taken 4 weeks following challenging were negative in sterility controls. As in fraction I-B re-

Table 7

Macroscopic Pathology and Cultural Results
in Group II-A Guinea Pigs

guinea pig number	sex	chal- lenged	Macroscopic Pathology							week of death
			wt.	spleen	liver	heart	lung	kidney	reproductive organs	
21	M	no	0.5	sl th nod*	n	n	n	n	cong testes	PM
22	M	yes	1.9	sl th nod	1 abs	fatty deposit	mar cong	n	cong testes pus	PM
23	M	no	0.7	n	n	n	n	n	n	PM
24	M	yes	0.8	sl th	n	n	sl cong	n	cong testes	PM
25	M	no	0.7	sl nod	n	n	n	n	n	PM
26	M	no	0.5	n	n	n	n	n	n	31
27	M	yes	0.7	sl th nod	n	n	mar cong	n	sl cong	PM ²
28	M	yes	1.0	n	n	n	n	n	n	PM
29	M	no	1.0	sl nod	n	n	n	n	sl cong	PM
30	M	yes	1.2	sl th nod	n	n	sl cong	n	n	PM ²

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

¹Not cultured, decomposed. No changes other than post mortem seen.

²Marked inflammation on back.

Key:

sl - slight	mod - moderate	nod - nodular
abs - abscess	preg - pregnant	th - thickened
mar - marked	n - normal	cong - congested

Table 8

Cultural and Pathological Results in Guinea Pigs Inoculated with
Challenge Used for Groups II-A and III-A

guinea pig number	sex	Macroscopic Pathology							
		spleen	liver	heart	lung	kidney	reproductive organs	week of death	
		wt. gms. pathology							
31	M	1.0	th abs*	abs*	n	cong	n	testes cong	PM
32	M	0.8	sl th nod*	abs*	n	cong	n*	cong	PM
33	M	0.6	th nod*	abs*	n	cong	n	cong pus filled*	PM

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

Key:

sl - slight
abs - abscess

th - thickened
n - normal

nod - nodular
cong - congested

Table 9

Macroscopic Pathology and Cultural Results
in Group II-B Guinea Pigs

guinea pig number	sex	chal- lenged	Macroscopic Pathology							week of death
			wt.	spleen gas. pathology	liver	heart	lung	kidney	reproductive organs	
111	M	no	1.0	th nod	abs	n	n	n	n	PM
112	M	no	0.4	n	n	n	n	n	n	PM
113	M	no	0.5	n	n	n	n	n	n	PM
114	F	yes	4.5	mar th enl nod*	abs*	n	n	n	early preg	PM
115	F	no	0.6	sl nod	few abs	n	n	n	live birth	PM
116	F	yes	5.7	mar th nod abs*	abs*	n	n	n	early preg	PM
117	M	no	1.5	sl th nod	n	n	n	n	sl cong testes	PM
118	M	no	1.5	sl th nod	n	n	sl cong	n	n	PM
119	M	no	1.3	sl th nod	few abs	n	mod cong	n	mod cong testes	PM
120	M	no	0.7	sl th	n	n	sl cong	n	sl cong testes	PM
131	F	no	-	n	n	n	pneumo	n	cong uterus	1 ^a
132	F	yes	3.9	th nod abs*	abs yellow	n	cong	n	2 wks preg	PM
133	M	yes	2.3	th nod abs*	abs*	n	cong	n	sl cong testes	PM
134	F	yes	5.0	th nod abs*	num abs*	n	n	n	early preg*	PM
135	M	yes	2.8	th nod*	abs	n	cong	n	cong testes	PM
136	M	yes	5.0	th nod*	abs enl*	n	n	n	n	PM
137	M	yes	2.2	sl nod*	abs	n	n	n	n	PM
138	F	yes	2.5	th nod*	few abs	n	n	n	early preg	PM
139	F	yes	1.5	sl th nod*	n	n	n*	n	3-4 wks preg	PM
140	M	no	-	n	n	n	pneumo	n	cong testes	1 ^a

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

^aDeath caused by pneumonia.

Key:

sl - slight
abs - abscess
mar - marked

mod - moderate
preg - pregnant
n - normal

nod - nodular
th - thickened
cong - congested

duced serological activity was evident though antibodies were produced in guinea pigs. Urease activity was absent.

Three inoculations at 10 day intervals of fraction II-C in 20 guinea pigs, 10 of which were subsequently challenged with 700 Br. abortus 2308 produced variable results both in production of agglutinin titers and in immunizing capacity. Four weeks following the challenge dose the sterility control animals still maintained a mean titer of 1:5 while challenged test animals and infection controls showed increased mean titers of 1:20 and 1:50 respectively. One of the infection controls (table 6) was not infected (animal G) and it is considered likely that both titers and infection of the controls would have been more severe in the absence of the sulfaguanidine these animals had been receiving. Two of the infection controls (animals E and F) from which organisms were recovered showed no evidence of titer in 1:25 dilutions of serum. However, in spite of the very low challenge dose 5 of the animals given II-C and challenged showed distinct rises in titers and Brucella were recovered from the spleens, which had a mean weight of 3.9 gm, and some of the other organs as shown in table 10. Guinea pig number 429 showed Brucella in cultures from the spleen and liver though no titer was evident. The absence of organisms on culture combined with negative titers indicated freedom from infection in the remaining 4 challenged animals. These had a mean spleen weight of 1.03 compared with 1.22 gm for the infection controls (table 6) and 0.9 in the unchallenged sterility controls. The spleens in both challenged

Table 10

Macroscopic Pathology and Cultural Results
in Group II-C Guinea Pigs

guinea pig number	sex	chal- lenged	Macroscopic Pathology								week of death
			spleen wt.gms. pathology	liver	heart	lung	kidney	reproductive organs			
411	M	yes	1.0	sl th nod*	num sm abs	n	mod cong	n	mar cong testes	PM ¹	
412	F	yes	10.0	mar th nod*	abs enl*	n*	mod cong	n*	6-7 wks preg*	PM	
413	M	yes	0.7	sl th nod*	few abs	n	sl cong	n	mod cong testes	PM	
414	F	no	0.6	n	abs	n	mod cong	n	n	2	
415	M	yes	6.9	mar th nod abs*	few abs	n	mod cong	n	mar cong testes	PM	
416	F	yes	1.3	sl th nod	few abs	n	mod cong	n	2-3 wks preg	PM	
417	M	yes	1.0	sl nod	n	n	n	n	sl cong	PM	
418	M	no	0.8	sl th nod	n	n	sl cong	n	mod cong testes	PM	
419	M	no	0.9	sl th nod	1 abs	n	sl cong	n	mod cong testes	PM	
420	M	no	0.8	n	n	n	n	n	n	PM	
421	M	no	0.6	sl nod	n	n	sl cong	n	sl cong testes	PM	
422	F	no	1.3	sl th	n	n	sl nod	n	2-3 wks preg	2	
423	F	no	1.1	sl th nod	n	n	sl nod	n	live birth	PM	
424	F	no	1.7	sl th	n	n	atrophy	n	2-3 wks preg	PM	
425	F	no	1.2	sl th	n	n	atrophy	n	early preg	PM	
426	M	yes	1.0	sl th nod	n	n	mod cong	n	n	PM	
427	F	no	0.5	n	n	n	mod cong	n	3-4 wks preg	PM	
428	M	yes	0.9	n	n*	n	mod cong	n	testes sl cong	PM	
429	M	yes	1.0	sl th nod*	n*	n	sl cong	n	n	PM	
430	M	yes	1.5	sl th	n	n	n	n	mod cong testes	PM	

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

¹Marked allergic reaction on hip.

Key:

sl - slight	mod - moderate	nod - nodular
abs - abscess	preg - pregnant	th - thickened
mar - marked	n - normal	cong - congested
num - numerous	sm - small	

and unchallenged animals showed some nodulation.

Fraction III. All animals inoculated with fractions III-A and III-C showed high titers and cultural and pathological evidence of infection, showing that Br. abortus 2308 survives prolonged treatment with acetone at low temperatures. Tables 11 and 12 show the results obtained in the 2 groups of animals. It had been wrongly assumed that Brucella would not survive acetone treatment and therefore there were no cultures made of the cell residue after such treatment. Three weeks following injection of fraction III-A, half of the 10 guinea pigs receiving it were given 1.0 ml amounts of the same challenge dose that was given group II-A, or 8,200 Br. abortus 2308. The other 5 animals which had received the fraction were sacrificed and cultural examination showed Brucella to be present. The spleens of these had a mean weight of 3.14 gm and pathology as shown in table 11 was typical of Brucella infection. Mean agglutinin titers for the group were about 1:370 at this time. Four weeks later, following challenging, the titers in the 5 remaining animals averaged 1:2500 and the spleens averaged 5.1 gm. The challenge dose was omitted in the 10 guinea pigs inoculated with III-C and only the titer 8 weeks following inoculation was taken. All 10 animals had a titer of more than 1:2000, the mean spleen weight was 5.95 gm and cultures demonstrated Brucella in all of the animals.

Fraction IV. A challenge dose of about 10,000 organisms was given 10 days after the 3 injections of fraction IV-B at 9 and 7 day intervals to half of the 20 guinea pigs inoculated

Table 11

**Macroscopic Pathology and Cultural Results
in Group III-A Guinea Pigs**

guinea pig number	sex	chal- lenged	Macroscopic Pathology								week of death
			spleen wt. gms.	pathology	liver	heart	lung	kidney	reproductive organs		
11	M	no	1.6	mod th nod*	abs*	n	cong*	n*	sl cong testes	PM	
12	M	yes	6.5	mar th nod abs*	num abs*	abs	mod cong	abs*	swollen pus- filled testes*	PM	
13	M	no	3.7	mar th nod*	abs*	n*	mar cong*	n	cong testes*	PM	
14	F	yes	2.8	mar th nod abs*	num abs*	n	mod cong	n	early preg	PM ¹	
15	M	no	5.0	mar th nod abs*	num abs*	n	cong	abs*	sl cong testes*	PM	
16	M	no	1.1	mod th nod*	num abs	n	cong	n	cong testes	PM	
17	M	yes	4.6	mar enl abs th nod*	num abs*	n	cong	n	cong testes*	PM	
18	M	yes	9.9	mar th abs nod*	abs*	surf abs	mar cong*	abs*	abs enl testes*	PM	
19	M	yes	1.7	mod th*	abs*	n	cong	n	sl cong testes	PM	
20	M	no	4.3	mar th nod abs*	num abs*	n	cong	n	cong testes	PM	

*Brucella recovered on culture. All other organs cultured and were negative for Brucella

¹Left forepaw swollen.

²Large supurating subdermal abscesses in axillary folds.

Key:

sl - slight
mod - moderate
nod - nodular
abs - abscess
preg - pregnant

th - thickened
mkd - marked
n - normal
cong - congested
surf - surface

Table 12

Macroscopic Pathology and Cultural Results
in Group III-C Guinea Pigs

guinea pig number	sex	Macroscopic Pathology							week of death
		spleen wt.gms.	pathology	liver	heart	lung	kidney	reproductive organs	
401	M	1.1	sl th nod*	n*	n	cong consol	n	mod cong testes*	PM ¹
402	F	6.4	mar th nod abs*	enl num abs*	n	consol*	n*	early preg ²	PM
403	F	12.4	mar th nod abs*	enl num abs*	n	cong	n*	2-3 wks preg*	PM
404	M	6.7	mar th nod abs*	enl num abs*	n	n	n	mod cong testes	PM
405	F	2.3	mar th nod abs*	num abs*	n	sl cong	n	aborted once early preg at	PM
406	M	8.5	mar nod th abs*	num abs*	n	mod cong	sl.enl n		PM
407	F	10.2	mar th nod abs*	num abs enl*	n	cong	n	aborted once early preg at	PM
408	F	6.0	mar th nod abs*	num abs*	n*	cong	n	4 wks preg*	PM
409	M	5.2	mar th nod abs*	num abs*	enl*	cong*	n	sl cong testes	PM
410	M	2.0	th enl nod*	num abs*	n	cong	n*	sl cong testes	PM

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

¹Left forepaw arthritic.

²Abscess at site of inoculation

Key:

No animals in this group challenged.

sl - slight
mod - moderate
nod - nodular
abs - abscess
preg - pregnant

th - thickened
mkd - marked
n - normal
cong - congested

and 4 infection controls. Four weeks following challenge all of the challenged animals had titers in excess of 1:200 and 3 of the 4 infection controls showed titers of at least 1:2000.

The mean weight of the spleens of the challenged animals (table 13) was 2.35 gm compared to a mean weight of 4.03 gm in the infection controls used for this group and groups VI-B and VII-B. Among the unchallenged animals the mean weight of the spleens was 1.1 gm. Some nodulation was apparent in 6 of the spleens of the unchallenged group but 4 appeared quite normal. Results on infection controls are in table 22.

Fraction V-1. Fraction V-1-B apparently protected 3 of 9 guinea pigs from infection while fraction V-1-C may have protected 7 of 10 animals. In the former case, all of 5 infection controls were infected while in the latter case one of 5 infection controls was not infected. None of 20 unchallenged animals, half given each fraction but not challenged, were infected.

A single inoculation of fraction V-1-B was given intraperitoneally and 4 weeks later 7,600 organisms were given by the same route as a challenge dose. From 3 animals, numbers 352, 354, and 357 as shown in table 14, no organisms were recovered. Absence of agglutinating antibodies in their sera supported the result.

Fraction V-1-C was given 20 guinea pigs in three 1 ml intraperitoneal injections spaced 10 days apart. A challenge dose of 14,000 organisms was given half of the animals 10 days after the last injection of fraction. Organisms were recovered

Table 13

**Macroscopic Pathology and Cultural Results
in Group IV-B Guinea Pigs**

guinea pig number	sex	chal- lenged	Macroscopic Pathology								week of death
			spleen wt. gms.	pathology	liver	heart	lung	kidney	reproductive organs		
501	F	yes	1.0	th nod abs*	n	n	sl cong	n	3 wks preg*	PM	
502	M	yes	6.1	th nod abs*	num sm	abs*	n consol adh*	abs enl*	mod cong testes*	PM	
503	F	yes	1.3	sl th nod*	num abs	n	n	n	2-3 wks preg	PM	
504	M	yes	1.6	mod th nod*	num abs enl	n	consol areas	enl	mod cong testes	PM	
505	M	yes	4.0	mar th nod*	num abs	n	sl cong	enl	mod cong testes	PM	
506	F	yes	1.9	mod th nod*	abs	n	sl cong	abs*	5-7 wks preg	PM	
507	M	yes	2.0	mod th nod abs*	abs	n	n	n	mod cong testes	PM	
508	F	yes	1.8	th nod abs*	abs*	n	mod cong	n	2 wks preg	PM	
509	M	yes	1.5	sl th nod*	abs	n	mod cong	n	sl cong testes	PM	
510	M	yes		decomposed	not done						
511	M	no	1.2	sl nod	n	n	sl cong	n	n	PM	
512	M	no	1.0	sl th nod	n	n	mod cong	n	sl cong testes	PM	
513	M	no	1.3	n	n	n	sl cong	n	n	PM	
514	F	no	0.8	n	n	n	n	n	5-6 wks preg	PM	
515	F	no	1.0	sl nod	n	n	n	n	5-6 wks preg	PM	
516	F	no	1.0	sl nod	n	n	n	n	n	PM	
517	M	no	1.0	n	n	n	n	n	sl cong testes	PM	
518	F	no	0.7	n	n	n	sl cong	n	preg fullterm	PM	
519	F	no	1.1	sl nod	n	n	sl cong	n	3 wks preg	PM	
520	M	no	0.9	n	n	n	n	n	n	3 ^a	

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

^aDeath caused by hemorrhage.

Key:

sl - slight	mod - moderate	nod - nodular
abs - abscess	preg - pregnant	th - thickened
mar - marked	n - normal	cong - congested
consol - consolidated		adh - adhesions

Table 14

**Macroscopic Pathology and Cultural Results
in Group V-1-b Guinea Pigs**

guinea pig number	sex	chal- lenged	Macroscopic Pathology								week of death
			spleen wt.gms.	pathology	liver	heart	lung	kidney	reproductive organs		
341	M	yes	2.8	mar th nod*	few abs	n	mod cong	n*	n	PM	
342	M	no	1.1	sl th	few abs	n	degen	n	infl epididymus testes cong	5 ^a	
343	M	no	0.5	n	n	enl	degen nod	n	cong testes	1 ^a	
344	F	yes	1.1	sl th*	abs	n	mod cong*	n	early preg	PM	
345	M	no	1.2	n	abs	n	pneumo	n	cong testes	5 ^a	
346	M	no		decomposed -	neg. culture					1 ^a	
347	F	no	1.2	n	enl	n	cong	enl	2-3 wks preg	6 ^a	
348	F	yes	1.6	th nod abs*	abs*	n	mod cong	n	6-7 wks preg	PM	
349	M	no	0.5	n	n	n	mar cong	enl	cong testes	1 ^a	
350	M	no	0.4	n	n	n	cheesy pl fl	enl	sl cong testes	6 ^a 1	
351	M	yes	1.0	sl th nod	n	n	sl cong	enl	cong testes	PM	
352	M	yes	1.8	mod th nod	n	n	mod cong	enl	sl cong testes	PM	
353	M	yes	4.8	mar th nod*	many abs	n	mod cong*	n	sl cong testes	PM	
354	M	no	1.7	th enl	mottled	n	mucoid pl fl	mod enl	cong testes	5 ^a	
355	F	no	1.2	sl nod	few abs	n	mod cong	n	7-8 wks preg	PM	
356	M	no	2.5	few sm abs	abs	n	bloody thick exudate	n	cong testes	1 ^a	
357	F	yes	1.9	sl th nod	few abs	n	sl cong	n	aborted once early preg at	PM	
358	M	yes	1.2	th nod	enl num abs	n	n	n	n	PM	
359	F	yes	1.8	sl th nod*	n	n	mod cong	n	early preg	PM	
360	F	no	0.3	n	2 lg abs	enl ³	cheesy pl fl	n	2-3 wks preg	2 ^a	

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

^aDeath caused by pneumonia.

¹Streptococci demonstrated in smear and culture from
pleural fluid.

²Abscess 5 cm. in diameter. Micrococci recovered.

³Cheesy pericardial fluid.

Key:

sl - slight mod - moderate
nod - nodular abs - abscess
preg - pregnant th - thickened
mar - marked n - normal
cong - congested pl - pleural
fl - fluid

from 3 animals, numbers 458, 466, and 468 as shown in table 15. These animals had definite titers when sacrificed while none of those challenged and uninfected, or unchallenged, had any titer.

The spleens of the infected and challenged animals given V-1-B and V-1-C had mean weights of 2.35 and 1.13 gm respectively. Uninfected animals given the fraction and challenged had mean weights of 1.23 and 0.86 gms respectively while those given fraction only had weights of 1.20 and 0.69 gm. The mean weights of spleens from infection controls (tables 19 and 20) used for the two groups were 2.55 and 1.16 gms respectively. The pathologic picture was confused in these animals as several suffered from enteritis and some from pneumonia. Livers showed atypical yellowed patchy abscesses in several animals known to be suffering from enteritis and animals, supposedly normal, taken from the pool and sacrificed without any sort of treatment showed similar abscesses.

Fraction V-2. Fraction V-2-B apparently protected 2 of 9 guinea pigs while fraction V-2-C protected 7 of 10. The controls used for these groups were the same as those used for groups V-1-B and V-1-C and it is noted that 1 of the 5 infection controls used in the latter group was not infected. All of the 10 unchallenged animals used as sterility controls for each fraction were uninfected.

A single 2.0 ml dose of fraction V-2-B was given 20 guinea pigs intraperitoneally. Four weeks later a challenge dose of 7,600 organisms was given half the animals. Of those challenged, two, numbers 373 and 377, showed no evidence of infection from

Table 15

**Macroscopic Pathology and Cultural Results
in Group V-1-C Guinea Pigs**

guinea pig number	sex	chal- lenged	Macroscopic Pathology								reproductive organs	week of death
			wt.	spleen gms. pathology	liver	heart	lung	kidney				
451	F	no	0.7	sl th nod	old abs	n	n	n	2 wks preg	PM		
452	F	no	0.9	sl th nod	n	n	n	n	early preg	PM		
453	F	no	0.6	sl th nod	old abs	n	n	n	early preg	PM		
454	F	no	0.5	n	n	n	n	n	early preg	PM		
455	M	no	0.9	sl th	n	n	n	n	sl cong testes	PM		
456	F	no	0.8	sl nod	n	n	n	n	early preg	PM		
457	F	yes	0.6	sl nod	n	n	n	n	2-3 wks preg	PM		
458	F	yes	0.9	sl th nod*	n	n	n	n	3-4 wks preg	PM		
459	F	no	0.9	sl th nod	n	n	sl cong	n	n	PM		
460	F	yes	0.9	sl th nod	n	n	sl cong	n	4-5 wks preg	PM		
461	F	no	0.9	sl th nod	few abs	n	scar tissue abs	n	3-4 wks preg	PM		
462	F	no	0.4	n	n	n	n	n	2-3 wks preg	PM		
463	M	yes	1.0	sl th nod	n	n	n	n	n	PM		
464	F	yes	1.0	sl nod	n	n	n	n	n	PM		
465	F	yes	1.0	sl nod	n	n	n	n	n	PM		
466	M	yes	0.9	sl th*	1 sm abs	n	n	n	sl cong testes	PM		
467	M	no	0.5	sl th nod	abs	n	n	n	mod cong testes	PM		
468	F	yes	1.6	sl th*	few abs*	n	sl cong	n	early preg	PM		
469	F	yes	1.0	sl th	n	n	sl cong	n	early preg	PM		
470	M	yes	0.5	n	n	n	mkd cong	n	n	PM		

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

^aDeath caused by hemorrhage.

^bDeath of undetermined origin.

^cDeath caused by pneumonia.

Key:

sl - slight

abs - abscess

mkd - marked

mod - moderate

preg - pregnant

n - normal

nod - nodular

th - thickened

cong - congested

culture or pathology and these showed no agglutinin titer. Agglutinin response was definite in the infected challenged animals. Spleen weights shown in table 16 correlated with infection, the mean of the infected and challenged animals being 1.76 gm compared with 2.55 gm in the infection controls used. Uninfected challenged animals averaged 0.85 gm and the animals which had received the fraction only showed slightly thickened spleens which averaged 0.99 gm. These animals had no agglutinin titers detectable at the time of sacrificing.

Twenty guinea pigs inoculated with fraction V-2-C received 3 intraperitoneal inoculations of 1.0 ml each at 10 day intervals. Ten days following the last inoculation of fraction V-2-C 14,000 organisms were given 10 of the animals intraperitoneally and the animals sacrificed 4 weeks later. There were 3 challenged animals in which organisms were demonstrable, numbers 473, 476, and 483. These animals also demonstrated rising titers at the time the experiment was terminated. The mean spleen weights of the challenged and unchallenged animals receiving the fraction were 1.04 and 1.05 gm respectively (table 17). Broken down on the basis of infected and uninfected challenged animals the mean weights were 1.08 and 0.97 gm for each group. The infection controls had mean spleen weights of 1.16 gm (table 20). Some thickening and nodulation of the spleens was apparent in the unchallenged animals.

The difference in the mean spleen weights of groups V-1-B and V-2-B and those receiving the later batches of fraction, groups V-1-C and V-2-C might be attributed to the increased

Table 16

**Macroscopic Pathology and Cultural Results
in Group 7-2-B Guinea Pigs**

guinea pig number	sex	chal- lenged	Macroscopic Pathology								reproductive week of organs death
			spleen wt. gms.	pathology	liver	heart	lung	kidney			
361	M	no	1.1	sl th nod	few abs	n	sl cong	enl	sl cong testes	PM	
362	M	no	0.7	sl nod	few abs	n	adhesions ¹	enl	mod cong testes	PM	
363	M	no	1.2	sl th nod	n	n	sl cong	enl	n	PM	
364	F	yes	1.9	th nod*	enl abs	n	sl cong	enl	2-3 wks preg	PM	
365		disappeared									
366	M	no	1.0	sl nod	n	n	sl cong	n	n	PM	
367	M	no	0.5	n	n	n	mar cong	n	n	PM	
368	M	no	0.7	n	n	n	cong	enl	cong testes	2 ^a	
369	M	no	0.8	n	abs	n	th bloody exudate	n	mar cong testes ext hem testes	2 ^a	
370	F	no	0.9	n	abs	n	mod cong	enl	2-3 wks preg	5 ^b	
371	F	yes	4.3	mar nod abs*	mar abs	n	mod cong	sl enl	cong uterus live birth	PM	
372	M	yes	1.4	sl th nod*	enl abs	n	cong	n	inguinal abs 5 cm diameter*	PM	
373	F	yes	0.9	n	n	n	sl cong	n	aborted 2 fetus at 6-7 wks	PM	
374	F	yes	2.0	th nod*	n*	n	mod cong*	sl enl*	aborted 2 fetus at 6-7 wks ²		
375	F	yes	2.3	th nod*	num abs*	n	sl cong*	n*	aborted 2 fetus at 6-7 wks*	PM	
376	M	yes	1.4	sl th nod*	abs	n	sl cong	n	mod cong testes	PM	
377	M	yes	0.6	sl nod	n	n	sl cong	n	n	PM	
378	M	no	2.0	smooth	abs	n	th bloody exudate	n	cong testes	3 ^a	
379	M	yes	0.9	sl th nod*	n	n	sl cong*	n	n	PM	

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

¹One lobe degenerate.

²Fetuses yielded Brucella also.

^aDeath caused by pneumonia.

^bDeath of undetermined origin.

Key:

sl - slight

mod - moderate

nod - nodular

abs - abscess

preg - pregnant

th - thickened

mkd - marked

n - normal

cong - congested

Table 17

**Macroscopic Pathology and Cultural Results
in Group V-2-C Guinea Pigs**

guinea pig number	sex	chal- lenged	spleen		liver	heart	lungs	kidney		reproductive organs	week of death
			wt.	pathology				l lobe	consol.		
471	M	yes	1.0	sl nod	n	n	n	l lobe	n	sl cong testes	PM
472	M	yes	0.6	sl nod	old abs	n	n	sl cong	n	sl cong testes	PM
473	M	yes	1.0	sl nod*	few abs	n	n	n	n	n	PM
474	F	yes	1.2	sl th	2 abs	n	n	n	n	2-3 wks preg	PM
475	F	yes	1.3	sl th nod	abs	n	n	n	n	2-3 wks preg	PM
476	F	yes	1.0	sl th nod*	abs	n	n	n	n	1 wk preg	PM
477	M	no	0.6	n	abs	n	n	cong	n	sl cong testes	1 ^a
478	F	yes	0.9	sl th nod*	n*	n	n	n*	n	1-2 wks preg	PM
479	F	yes	1.3	sl th	few abs	n	n	sl cong	n	n	PM
480	F	no	0.7	sl th nod	n	n	n	sl cong	n	cong uterus	3 ^b
481	F	no	1.1	sl th nod	n	n	n	mod cong	n	n	PM
482	F	no	1.1	sl th nod	2 sm abs	n	n	n	n	n	PM
483	F	no	1.2	sl th nod	n	n	n	n	n	n	PM
484	F	no	1.6	mod th	few sm abs	n	n	n	n	n	PM
485	F	no	1.3	sl th nod	1 abs	n	n	n	n	n	PM
486	F	no	1.0	sl th nod	n	n	n	degen nod	n	cong. uterus	3 ^b
487	F	no	1.4	sl th nod	n	n	n	consol.	n	cong. uterus	2 ^c
488	F	no	1.2	sl th	n	n	n	cong.hem	n	cong. uterus	PM
489	F	yes	1.1	mod th	n	n	n	mod cong	n	n	PM
490	F	yes	1.1	sl th nod	few abs	n	n	n	n	n	PM

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

^aDeath caused by enteritis.

^bDeath caused by hemorrhage.

^cDeath caused by pneumonia.

Key:

sl - slight mod - moderate nod - nodular
abs - abscess preg - pregnant th - thickened
mkd - marked n - normal cong - congested

protective power resulting from 3 inoculating doses. However, a consideration of the drop in spleen weight of the infection controls (tables 19 and 20), in spite of a heavier infective dose combined with the failure of 1 animal to become infected suggests lowered infectivity. The cause of this is not known.

Fraction VI. Both preparations of fraction VI gave favorable results. Fraction VI-A protected 8 of 9 animals challenged while fraction VI-B protected 6 of 11 animals challenged. All of 9 infection controls used were infected.

A single intraperitoneal inoculation of 5.0 ml of fraction VI-A in each of 20 guinea pigs protected 8 of 9 subsequently challenged. None of 10 unchallenged animals was infected while 5 infection controls were. Agglutinin titers were absent in all animals receiving the fraction at the time of post mortem examination and the single animal which was challenged and infected had a practically normal spleen (table 18) and carried two 6 weeks fetuses. The mean spleen weights of infected and uninfected challenged animals were 1.1 and 1.1 gm respectively. Unchallenged animals receiving the fraction had a mean spleen weight of 0.9 gm. There was some thickening among these spleens and the surface was nodulated. The infection controls (table 19) had spleens averaging 2.55 gm.

Fraction VI-B given in three 2.0 ml intraperitoneal injections at 9 and 7 day intervals followed by a challenge dose of 10,000 organisms did not show as good protection. Five animals, numbers 523, 524, 525, 528, 529, and 535 were infected. These animals further had rather large spleens, the mean weight

Table 18

**Macroscopic Pathology and Cultural Results
in Group VI-A Guinea Pigs**

guinea pig number	sex	chal- lenged	Macroscopic Pathology								week of death
			wt.	spleen gms. pathology	liver	heart	lung	kidney	reproductive organs		
381	M	yes	1.6	sl nod th	abs	n	cong	sl enl	cong testes	PM	
382	M	yes	not recorded					consol		5 ^a	
383	F	yes	1.1	sl th*	n*	n	pneumo	n	6 wks preg	5 ^a	
384	F	yes	1.0	sl nod	n	n	n	n	early preg	PM	
385	F	yes	0.9	sl th	n	n	pneumo	n	2 live births before chall.	5 ^a	
386	F	no	3.1	enl th abs	few abs	n	pneumo	n	4 wks preg	PM	
387	F	no	2.5	pulpy abs th nod	enl	abs at site of bleeding	pneumo	n	early preg	6 ^a	
388	F	yes	1.3	sl th	mod enl	n	sl cong	n	early preg	PM	
389	M	yes	0.9	sl th nod	n	enl abs	pneumo	n	mar cong testes	6 ^a	
390	M	no	-	n	n	n	n	n	n	PM	
391	F	yes	1.0	sl nod	n	n	sl cong	n	2-3 wks preg	PM	
392	M	yes	0.6	sl nod	few abs	n	n	n	n	PM	
393	M	no	0.5	n	mottled	cheesy fl. in	cheesy fl. in pericardium	n	cong testes	1 ^a	
394	F	no	1.7	abs 1 cm in diameter	n	n	cheesy fl in pleurae	n	cong uterus	4 ^a	
395	M	no	decomposed					cheesy fl in pleurae		4 ^a	
396	M	no	0.4	n	n	n	pneumo	n	n	4 ^a	
397	M	no	0.9	sl nod	abs	n	sl cong	n	sl cong testes	PM	
398	M	no	1.2	sl nod	n	n	sl cong	n	sl cong testes	PM	
399	M	no	0.9	n	abs	n	sl cong	n	n	PM	
400	M	no	0.9	n	n	n	sl cong	n	cong testes	PM	

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

^aDeath caused by pneumonia.

Key:

sl - slight	mod - moderate	nod - nodular
abs - abscess	preg - pregnant	th - thickened
mar - marked	n - normal	cong - congested

Table 19

Cultural and Pathological Results in Guinea Pigs Inoculated with
Challenge Used for Groups V-1-B,
V-2-B and VI-A

guinea pig number	sex	Macroscopic Pathology					week of death
		wt. gms.	spleen pathology	liver	heart lung	kidney reproductive organs	
1	M	4.3	mkd th abs*	abs*	n	mod cong	n* sl cong ¹ PM
2	F	3.5	mkd th nod*	abs enl*	n	mod cong	n 2 wk preg ¹ PM
3	H	1.0	mod th nod*	n*	n	mod cong	n* sl cong PM
4	F	1.4	sl th*	few abs*	n	mod cong	n* 2 live young PM
5	F	0.6	n	abs	n	mkd cong pneumo	n* 1 wk preg 1

*Brucella recovered on culture. All other organs cultured and negative for brucella.

¹Right hind leg abscessed.

Key:

sl - slight th - thickened nod - nodular
abs - abscess n - normal cong - congested
preg - pregnant mkd - marked

Table 20

**Cultural and Pathological Results in Guinea Pigs Inoculated with
Challenge Used for Groups V-1-C and V-2-C**

guinea pig number	sex	Macroscopic Pathology								
		spleen		liver	heart	lung	kidney	reproductive organs	week of death	
		wt.gms.	pathology							
O	M	1.2	sl th nod*	few abs*	n	sl cong*	n*	n	PM	
P	F	1.4	sl th nod*	n	n	sl cong	n	uterus cong	PM	
Q	M	1.2	sl th nod	few abs	n	sl cong	abs	n	PM	
R	M	0.9	sl th nod*	n	n	n	n	n	PM	
S	F	1.6	sl th nod*	few abs*	n	sl cong	n*	n	PM	

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

Challenge dose 14,000 organisms.

Key:

sl - slight	th - thickened	nod - nodular
abs - abscess	n - normal	cong - congested

of which was 2.40 gm. Two, numbers 523 and 529, in particular had exceptionally large spleens, which weighed 5.1 and 3.1 gm each (table 21). The spleens of infection controls (table 22) were even larger, averaging 4.03 gm. All infected animals demonstrated titers, though the mean of the animals receiving the fractions and then challenged was 1:90 as opposed to the titers of the infection controls which were over 1:200. Non infected animals which had been challenged, numbers 522, 526, 527, 530, and 531, had a mean spleen weight of 1.16 while the unchallenged animals receiving the fraction had a mean spleen weight of 0.87. As was the case with fraction VI-A, the spleens of these animals showed some thickening and nodulation. The lowered spleen weights compared with the infection controls probably gives a valid indication of modification of the disease.

Fraction VII. No indication of protection was obtained in 10 guinea pigs inoculated with fraction VII and subsequently challenged with 10,000 organisms. A single intraperitoneal inoculation of 5.0 ml of fraction was given 20 guinea pigs in all. Half of these were challenged and all animals sacrificed 30 days after the challenge dose was given. The mean spleen weight of the infected challenged animals (table 23) was 1.83 gm while that of the infection controls was 4.03 gm (table 22). Unchallenged animals had a mean spleen weight of 1.03 gm and the spleens showed thickening and nodulation. The only possible evidence of protection was the lower spleen weight of the infected test animals when compared to that of the infection controls. Agglutination titers in the infected animals

Table 21

Macroscopic Pathology and Cultural Results
in Group VI-B Guinea Pigs

guinea pig number	sex	chal- lenged	Macroscopic Pathology								week of death
			wt.gms.	spleen pathology	liver	heart	lung	kidney	reproductive organs		
521	M	no	1.1	sl th nod	abs	n	n	n	mod cong testes	PM	
522	F	yes	1.4	sl th nod	n	n	n	n	4-5 wk preg	PM	
523	F	yes	5.1	mar th nod*	num abs*	n	sl cong*	n*	2-3 wk preg	PM	
524	F	yes	2.1	th nod*	abs*	n	sl cong	abs*	1-2 wk preg	PM	
525	M	yes	1.2	sl th nod*	abs	n	sl cong	n	sl cong testes	PM	
526	M	yes	0.9	n	n	n	n	n	n	PM	
527	M	yes	1.3	sl th nod	abs	n	n	n	sl cong testes	PM	
528	M	yes	1.6	sl th nod*	abs	n	sl cong	n	sl cong testes	PM	
529	F	yes	3.1	mar th nod*	few abs*	n	cong	n	4 wks preg	PM	
530	M	yes	0.9	sl th	n	n	n	n	n	PM	
531	F	yes	1.3	sl nod	n	n	n	n	2-3 wk preg	PM	
532	M	no	0.8	sl nod	abs	n	n	sl enl	mod cong testes	PM	
533	M	no	0.7	sl nod	abs	n	sl cong	n	sl cong testes	PM	
534	M	no	0.8	sl th nod	n	n	sl cong	n	sl cong testes	PM	
535	M	yes	1.1	n*	abs*	n	n*	n*	n	PM	
536	M	no	0.8	sl nod	n	n	n	sl enl	sl cong testes	PM	
537	M	no	0.8	sl nod	n	n	sl cong	n	n	PM	
538	M	no	1.1	sl th nod	n	n	n	sl enl	sl cong testes	PM	
539	M	no	0.7	sl nod	n	n	n	n	n	PM	
540	M	no	0.7	sl nod	n	n	n	n	n	PM	

*Brucella recovered on culture. All other organs cultured and were negative for Brucella.

Key:

sl - slight
mod - moderate
nod - nodular
abs - abscess

preg - pregnant
th - thickened
mkd - marked
n - normal
cong - congested

Table 22

Cultural and Pathological Results in Guinea Pigs Inoculated with
Challenge Used for Groups VI-B and VII-B

Guinea pig number	sex	Macroscopic Pathology					week of death
		wt. gms.	spleen	liver	heart	lung	kidney reproductive organs
T	M	1.0	sl th nod*	abs enl*	n	sl cong*	n n PM
U	F	2.6	nod th nod*	abs enl*	n	sl cong*	n 3 wks preg PM
V	M	3.2	mkd th enl*	abs enl*	n	cong*	enl* epididymus and testes inflamed* PM
W	M	3.3	mkd th nod*	abs*	n	sl cong	n epididymus pus filled PM

*Brucella recovered on culture. All other organs cultured and negative for Brucella.

Key:

sl - slight th - thickened nod - nodular
abs - abscess n - normal cong - congested
preg - pregnant mkd - marked

Table 23

Macroscopic Pathology and Cultural Results
in Group VII-B Guinea Pigs

guinea pig number	sex	chal- length	Macroscopic Pathology					kidney	reproductive	week of death
			wt.	spleen	liver	heart	lung			
				pathology				organs		
541	M	no	1.2	nod	old abs	n	sl cong	n	sl cong testes	PM
542	M	no	0.7	sl th nod	old abs	n	sl cong	n	mod cong testes	PM
543	F	no	1.1	sl th nod	old abs	n	sl cong	n	1 wk preg	PM
544	M	no	0.8	n	1 abs	n	sl cong	n	sl cong testes	PM
545	M	no	1.0	sl th	n	n	n	n	sl cong testes	PM
546	M	no	0.7	sl nod	abs	n	sl cong	n	sl cong testes	PM
547	M	no	1.5	sl th nod	n	n	n	n	mod cong testes	PM
548	M	no	1.5	th nod	n	n	n	n	sl cong testes	PM
549	M	no	0.8	sl nod	n	n	n	n	sl cong testes	PM
550	M	no	1.0	sl th nod	n	n	n	n	sl cong testes	PM
491	F	yes	2.5	mod th nod*	old abs*	n	sl cong*	n	recent abortion	PM
492	M	yes	1.2	sl th nod*	n	n	sl cong	n	sl cong testes	PM
493	M	yes	0.7	sl th nod*	n	n	nod cong	n	sl cong testes	PM
494	M	no	-	*	n	n	*	n	n	PM
495	F	no	1.0	sl th nod*	few abs	n	n	n	n	4a
496	F	yes	3.2	mkd th nod*	n*	n	n	n	n not preg	1
497	M	yes	3.2	mkd th nod*	abs	n	sl cong	n	n	PM
498	F	yes	1.5	sl th nod*	n	n	n	n	n not preg	PM
499	M	yes	0.6	n*	few abs	n	n	n	n	PM
500	M	yes	0.5	n*	n	n	n	n	n	7a

*Brucella recovered. If no other indication organ was cultured and no Brucella found.
aDeath caused by enteritis.

Key:

sl - slight
mod - moderate
nod - nodular
abs - abscess
preg - pregnant

th - thickened
mkd - marked
n - normal
cong - congested

were over 1:200 and therefor gave no evidence of a modified infection.

In Vivo Antibody Production

Antibody response in guinea pigs receiving single inoculations of fractions was, in general, slight. However, the 3 to 4 weeks elapsing between inoculations and titering was probably too long a period to permit observation of many low titers. The animals inoculated with fraction I-A are not considered inasmuch as the sterility controls were infected by the fraction itself.

The water extracted fractions VI-A and I-B produced only partial agglutination in dilutions of 1:25 in 3 of 20 guinea pigs each as shown in tables 24 and 25 where titers taken 4 weeks after a single inoculation of fraction are presented. Only 1 guinea pig, number 396, inoculated with fraction VI-A showed a complete agglutination in 1:25 dilution. No tests with a single inoculation of fraction VII were done.

Among the guinea pigs receiving the 30 per cent acetone soluble fraction V-1-B, only partial agglutinations in 1:25 to 1:50 dilutions were shown in 3 of 20 guinea pigs 4 weeks after administration of the fractions. Among those receiving the 30 per cent acetone insoluble fraction V-2-B only 2 partial agglutinations in 1:25 dilution were seen in 20 guinea pigs. Tables 26 and 27 show the results.

Titers were more definite in animals receiving fraction II-A and II-B, most of the guinea pigs receiving fraction II-A demonstrating complete agglutination in 1:25 dilutions after

Table 24

Titers Produced from 1 Inoculation of Fraction VI-A
and a Challenge Dose in Guinea Pigs

guinea pig number	challenged	infected	titer 4 weeks after fraction		titer 4 weeks after challenge	
			complete	partial	complete	partial
381	yes	no	-	-	-	-
382	yes	no	-	+ 1:25	dead	-
383	yes	yes	-	-	dead	-
384	yes	no	-	-	-	-
385	yes	no	-	+ 1:25	dead	-
386	no	no	-	-	-	-
387	no	no	-	-	dead	-
388	yes	no	-	-	-	-
389	yes	no	-	-	dead	-
390	no	no	-	-	-	-
391	yes	no	-	3+1:25	-	-
392	yes	no	-	-	-	-
393	no	no	dead	-	-	-
394	no	no	-	-	dead	-
395	no	no	-	-	dead	-
396	no	no	1:25	-	dead	-
397	no	no	-	-	-	-
398	no	no	-	-	-	-
399	no	no	-	-	-	-
400	no	no	-	-	-	-

Table 25

Antibody Titers in Guinea Pigs 30 Days Following Inoculation of
Fraction I-B and 30 Days Following Challenge Dose of
100,000 Br. abortus 2308

guinea pig number	challenged	infected	titers produced by inoculation of	titers produced following inoculation			
			fraction partial only	with 100,000 <u>Br. abortus</u> after 14 days		after 30 days	
				complete	partial	complete	partial
101	no	no	-	-		-	
102	no	no	-	-		-	
103	no	no	+ 1:25	-		-	
104	no	no	-	-		-	
105	no	no	-	-		-	
106	no	no	-	-		-	
107	no	no	-	-		-	
108	no	no	-	-		-	
109	no	no	-	-		-	
110	no	no	-	-		-	
121	yes	yes	-	1:100	3+1:200	1:200	2+1:250
122	yes	yes	-	1:100	2+1:200	1:200	3+1:250
123	yes	yes	-	1:25	+1:100*	1:500	1+1:2000
124	yes	yes	1+1:50	1:250	3+1:500	1:200	1+1:500
125	yes	yes	1+1:50	1:250	1+1:500	dead	
126	yes	yes	-	1:100	-	1:2000 ¹	
127	yes	yes	-	1:100	1+1:250	1:1000	2+1:2000
128	yes	yes	-	1:200	+1:250*	1:500	3+1:1000
129	yes	yes	-	1:500	-	1:1000	2+1:1000
130	yes	yes	-	1:100	+1:200*	1:500	3+1:1000

*partial titers are given for the last tube showing any agglutination

¹titer not carried beyond 1:2000

Table 26

Titers Produced from 1 Inoculation of Fraction V-1-B
and a Challenge Dose in Guinea Pigs

guinea pig number	challenged	infected	titer 4 weeks after fraction		titer 4 weeks after challenge	
			complete	partial	complete	partial
341	yes	yes	-	-	1:500	1+1:1000
342	no	no	-	-	dead	
343	no	no	dead			
344	yes	yes	-	-	1:25	1+1:50
345	no	no	-	-	dead	
346	no	no	dead			
347	no	no	-	-	dead	
348	yes	yes	-	-	1:250	3+1:500
349	no	no	dead			
350	no	no	-	-	dead	
351	yes	no	-	-	-	-
352	yes	no	-	-	-	-
353	yes	yes	-	+ 1:25	1:1000	3+1:2000
354	no	no	-	-	dead	
355	no	no	-	-	-	-
356	no	no	dead			
357	yes	no	-	+ 1:25	-	-
358	yes	yes	-	+ 1:25	1:200	-
359	yes	yes	-	-	-	+ 1:25
360	no	no	dead			

Table 27

Titers Produced from 1 Inoculation of Fraction V-2-B
and a Challenge Dose in Guinea Pigs

guinea pig number	challenged	infected	titer 4 weeks after fraction		titer 4 weeks after challenge	
			complete	partial	complete	partial
361	no	no	-	+ 1:25	-	-
362	no	no	-	-	-	-
363	no	no	-	-	-	-
364	yes	yes	-	-	1:1000	2+1:2000
365	no	no	-	-	gone	-
366	no	no	-	-	-	-
367	no	no	-	-	-	-
368	no	no	dead	-	-	-
369	no	no	dead	-	-	-
370	no	no	-	+ 1:50	dead	-
371	yes	yes	-	-	1:1000	2+1:2000
372	yes	yes	-	-	1:1000	-
373	yes	no	-	-	-	-
374	yes	yes	-	-	1:1000	-
375	yes	yes	-	-	1:1000	3+1:2000
376	yes	yes	-	-	1:25	-
377	yes	no	-	-	-	-
378	no	no	dead	-	-	-
379	yes	yes	-	-	1:100	2+1:500

3 weeks while most of those receiving fraction II-B showed partial agglutinations in dilutions of 1:25 to 1:50 after 4 weeks (tables 28 and 29).

Increasing the number of inoculations of fraction to 3 and making observation of titers at the time each inoculation was given, 10 days following the last inoculation of fraction, and 2 and 4 weeks respectively after the administration of the challenge dose gave more satisfactory measure of agglutinins in the animals. Figures 3 and 4 summarize the mean titers of challenged and unchallenged animals so treated.

Both the fractions extracted with alkaline solution (fraction VI-B) and neutral solution (fraction I-C) produced antibodies to a mean titer of about 1:40 as shown in figure 3. The method of calculating the mean titers is shown in appendix VI. Nearly all of the animals showed complete agglutination in 1:25 or 1:50 dilutions 10 days after inoculation of fraction I-C but the general tendency was downward in spite of 2 more inoculations at 10 day intervals. Fraction VI-B, on the other hand, showed a further increase to 1:50 following the second inoculation and a decline thereafter. This would seem to indicate a greater stability on standing in the alkaline extracted fraction than in that extracted in neutral solution. Fraction VII, extracted at low pH, did not stimulate antibodies in all guinea pigs inoculated. Compared to the alkaline and neutral water extracts it would seem to be the least stable as the titer from the first inoculation was lowest and 2 more inoculations failed to further increase the titer in any case.

Table 28

Agglutinin Response Following One Inoculation
of Fraction II-A and of Challenge

guinea pig number	challenged	infected	titer 3 weeks after fraction injected		titer 4 weeks after challenge	
			complete	partial	complete	partial
21	no	no	-	-	dead	
22	yes	no	1:50	+ 1:200	-	
23	no	no	1:25	-	dead	
24	yes	no	1:100	1+1:200	-	
25	no	no	1:25	1+1:100	dead	
26	no	no	dead		dead	
27	yes	no	1:100	1+1:250	-	
28	yes	no	1:50	1+1:100	-	
29	no	no	-	+ 1:25	dead	
30	yes	no	-	+ 1:25	-	

Table 29

Titers Produced from 1 Inoculation of Fraction XI-B
and a Challenge Dose in Guinea Pigs

Guinea chal- pigs	lenged	in- fectcd	titer 4		titer 2		titer 4	
			weeks after	fraction	weeks after	challenge	weeks after	challenge
number				compl. partial	compl. partial	compl. partial	compl. partial	partial
111	no	no	-	+ 1:50	-	-	-	-
112	no	no	-	-	-	-	-	-
113	no	no	-	-	-	-	-	-
114	yes	yes	-	+ 1:50	0	0	1:500	+ 1:1000
115	no	no	-	+ 1:1:50	-	-	-	-
116	yes	yes	-	+ 1:25	0	0	1:500	3+1:1000
117	no	no	-	+ 1:50	-	-	-	-
118	no	no	-	+ 1:25	-	-	-	-
119	no	no	-	+ 1:50	-	-	-	-
120	no	no	-	+ 1:25	-	-	-	-
131	no	no	-	-	-	-	-	-
132	yes	yes	1:50	-	1:250	+ 1:500	1:2000	1
133	yes	yes	-	+ 1:1:25	1:1000	+ 3:2000	1:2000	1
134	yes	yes	-	+ 1:1:50	1:1000	+ 1:2000	1:2000	1
135	yes	yes	-	-	1:200	-	1:1000	1+1:2000
136	yes	yes	-	+ 1:1:25	1:250	+ 1:500	1:2000	1
137	yes	yes	-	+ 1:1:25	1:1000	+ 1:2000	1:2000	1
138	yes	yes	-	+ 1:1:25	1:250	+ 1:500	1:2000	1
139	yes	yes	-	-	1:250	1+1:500	1:2000	1
140	no	no	dead	-	-	-	-	-

1 Titer not carried beyond 1:2000

Figure 3

Production of Agglutinins from Inoculation of
Water Soluble Fractions of Brucella abortus

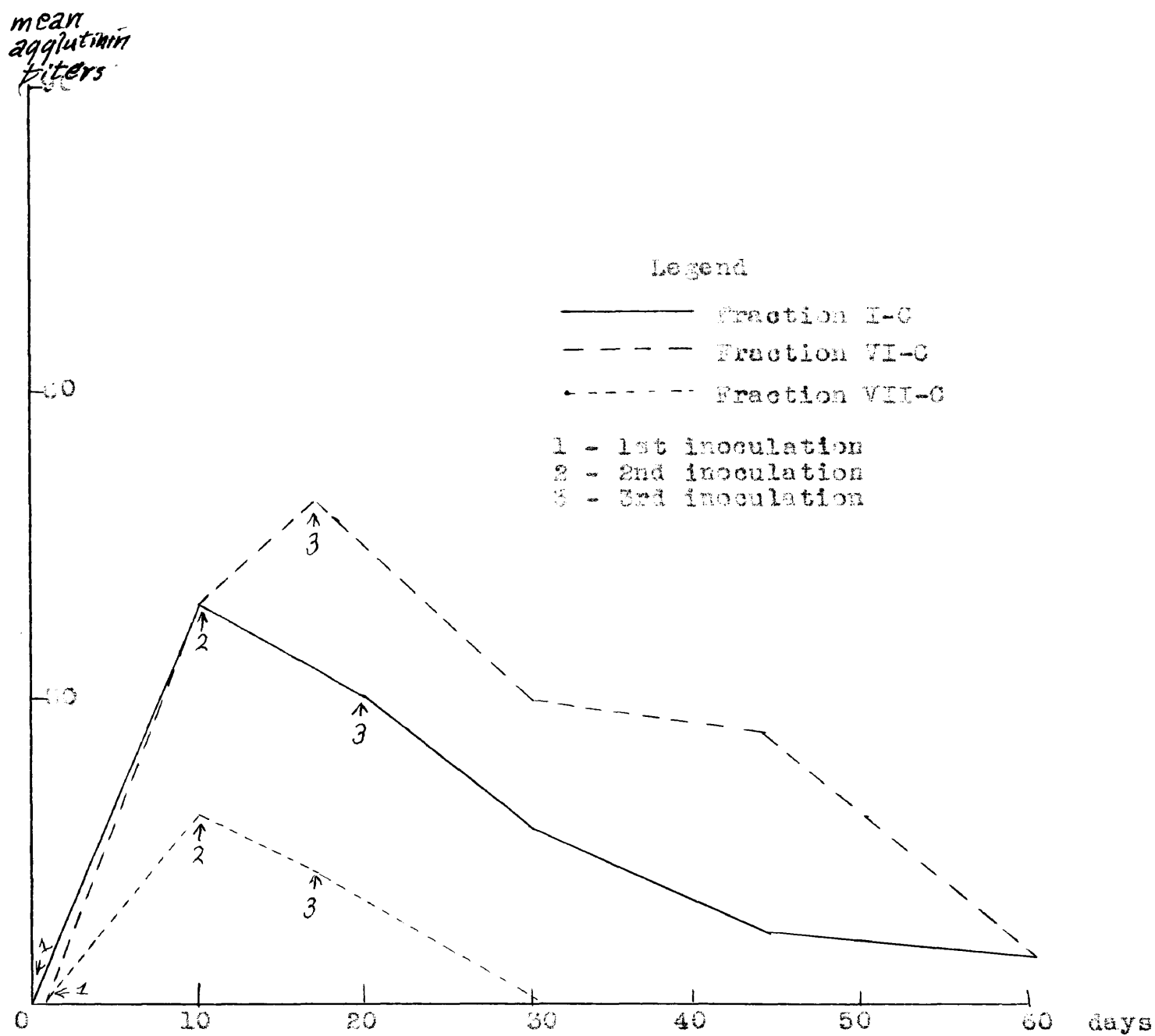
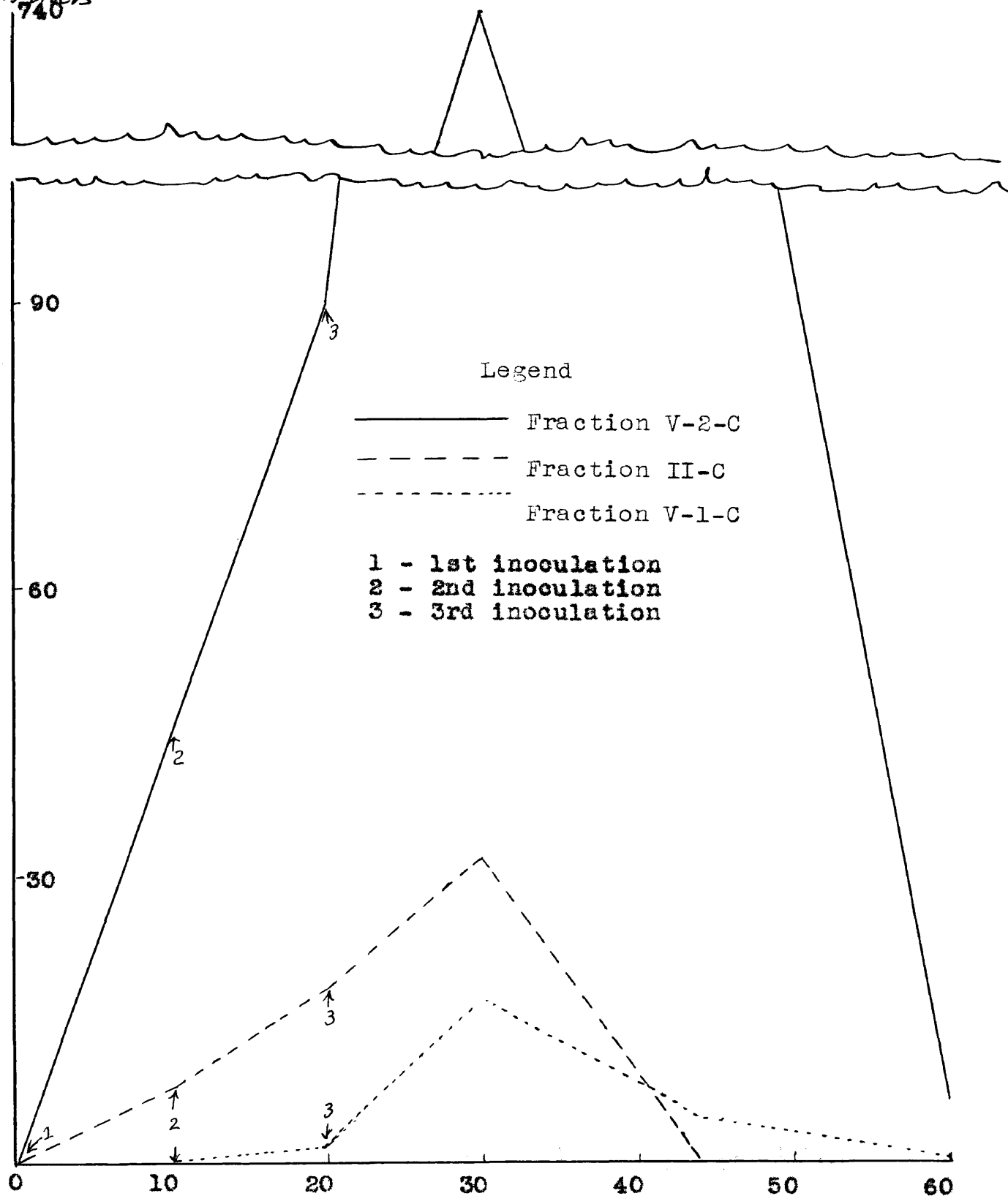


Figure 4

Production of Agglutinins from Inoculation of Acetone
Treated Fractions of Br. abortus

mean
agglutinin
titer



Since the alkaline and acid extracts were made from the same cell suspension the difference should be valid. All animals inoculated with fractions I-C and VI-B showed at least partial agglutination in 1:25 dilutions as shown in tables 30 and 31. But 3 of those receiving fraction VII-B failed to show partial agglutination in 1:25 dilution (table 32).

The agglutinin response in animals receiving 3 inoculations of the 30 per cent acetone soluble fraction V-1-C was very slow. Figure 4 shows that no mean titer was evident 10 days after the first inoculation and table 33 shows that no agglutinins were present in any animals. Ten days after the second inoculation 6 animals showed weak response in 1:25 to 1:50 dilutions as shown in table 33. Following the third inoculation the mean titer (figure 4) showed an increase. All but 5 animals showed some degree of agglutinin response. The response in the animals inoculated with fraction V-2-C was more striking. All animals responded as shown in table 34 with the mean titer 9 days following the first inoculation being 1:45, that 7 days following the second inoculation being about 1:90 and that 10 days following the third inoculation rising sharply to over 1:700. These are demonstrated graphically in figure 4.

Of the 2 fractions treated with concentrated acetone 3 inoculations of fraction IV-B failed to elicit antibody production in guinea pigs with 4 exceptions. In table 35 it is shown that titers in these were quite low. Three inoculations of fraction II-C, however, produced a low degree of agglutinin response in every case, as shown in table 36. In figure 3 it

Table 30

Titers Produced from 3 Inoculations of Fraction I-C
and a Challenge Dose in Guinea Pigs

guinea pig number	challenged	infected	Highest dilu- tion agglutin- ating 10 days after first inoculation ¹		Highest dilu- tion agglutin- ating 10 days after second inoculation		Highest dilu- tion agglutin- ating 10 days after third inoculation		Highest dilu- tion agglutin- ating 14 days after challeng- ing		Highest dilu- tion agglutin- ating 30 days after chal- lenging	
			compl.	partial	compl.	partial	compl.	partial	compl.	partial	compl.	partial
431	yes	no	1:50	+1:200	1:50	1+1:100	-	-	-	+ 1:25	-	-
432	yes	no	-	+ 1:25	-	+ 1:25	-	-	-	-	-	-
433	yes	no	1:50	+ 1:100	-	+ 1:50	-	-	-	-	-	-
434	yes	no	1:50	1+1:100	1:25	2+1:50	-	2+1:25	-	-	1:50	+ 1:100
435	yes	no	-	+ 1:25	-	+ 1:25	-	-	-	-	-	+ 1:25
436	yes	yes	1:50	+ 1:100	1:25	+ 1:50	-	-	-	-	-	+ 1:25
437	yes	no	1:50	1+1:100	-	+ 1:50	-	-	-	-	-	-
438	yes	no	1:25	1+1:50	1:25	+ 1:50	1:25	1+1:50	-	+ 1:50	-	3+1:25
439	yes	no	1:25	+ 1:50	1:50	+ 1:100	1:50	1+1:100	1:25	+ 1:100	1:25	-
440	yes	no	-	-	-	+ 1:25	-	-	-	-	-	-
441	no	no	-	+ 1:25	-	+ 1:25	-	-	-	-	-	+ 1:25
442	no	no	-	1+1:25	-	1+1:25	1:25	1+1:100	-	+ 1:50	1:25	1+1:25
443	no	no	1:25	+ 1:100	-	+ 1:25	-	-	-	-	-	+ 1:25
444	no	no	1:25	1+1:50	1:50	+ 1:100	-	+ 1:25	-	-	-	-
445	no	no	1:50	-	1:25	1+1:100	-	+ 1:25	-	-	-	-
446	no	no	1:25	1+1:50	1:25	3+1:50	-	+ 1:50	-	-	-	-
447	no	no	1:50	+ 1:100	-	+ 1:25	-	-	-	-	-	-
448	no	no	1:50	2+1:100	1:50	3+1:100	1:25	-	-	+ 1:25	-	-
449	no	no	1:50	1+1:100	1:50	3+1:100	1:50	+ 1:200	1:25	1+1:50	-	1+1:25
450	no	no	1:25	1+1:50	1:50	1:100	dead					

All titers negative in 1:25 dilution before first inoculation.

¹Blood samples taken and next inoculation given on same day.

²Challenge dose of 700 Br. abortus 2308 given 10 days after last inoculation of fraction.

Table 31

Titers Produced from 3 Inoculations of Fraction VI-B
and a Challenge Dose in Guinea Pigs

guinea pig number	challenged	infected	Highest dilu- tion aggluti- nating 9 days after first inoculation ¹		Highest dilu- tion aggluti- nating 7 days after second inoculation		Highest dilu- tion aggluti- nating 10 days after third inoculation		Highest dilu- tion aggluti- nating 14 days after chal- lenging ²		Highest dilu- tion aggluti- nating 30 days after chal- lenging	
			compl.	partial	compl.	partial	compl.	partial	compl.	partial	compl.	partial
521	no	no	1:50	1+1:100	1:50	3+1:100	1:25	2+1:50	-	-	-	-
522	yes	no	1:25	+ 1:100	1:25	+ 1:100	1:100	1+1:25	1:25	1+1:50	1:25	1+1:50
523	yes	yes	1:25	+ 1:100	1:100	+ 1:200	1:50	1+1:25	1:100	-	1:200 ³	-
524	yes	yes	-	+ 1:25	1:25	+ 1:50	-	+ 1:25	1:25	1+1:50	1:200 ³	-
525	yes	yes	1:50	+ 1:200	1:100	+ 1:200	1:25	2+1:100	-	+ 1:25	1:25	+ 1:50
526	yes	no	1:50	+ 1:200	1:100	1+1:200	1:25	2+1:50	-	+ 1:50	-	-
527	yes	no	1:50	2+1:100	1:100	1+1:200	-	-	-	-	-	-
528	yes	yes	-	1+1:50	1:25	+ 1:50	-	+ 1:50	-	-	-	+ 1:25
529	yes	yes	1:25	+ 1:100	1:25	1+1:50	1:25	1+1:100	1:50	+ 1:100	1:100	2+1:200
530	yes	no	1:25	+ 1:100	-	+ 1:25	-	1+1:25	1:25	+ 1:100	-	-
531	yes	no	1:25	+ 1:100	-	+ 1:50	1:50	3+1:100	-	-	1:25	1+1:50
532	no	no	1:25	+ 1:100	1:50	1+1:100	-	-	-	-	-	-
533	no	no	-	+ 1:25	1:25	+ 1:100	1:50	+ 1:100	-	-	-	-
534	no	no	1:25	+ 1:100	-	+ 1:25	0	0	1:25	+ 1:100	1:25	+ 1:50
535	yes	yes	-	1+1:50	-	+ 1:25	-	-	1:25	+ 1:100	1:200 ³	-
536	no	no	-	+ 1:100	-	+ 1:25	-	+ 1:25	-	-	-	-
537	no	no	-	+ 1:50	1:25	+ 1:100	-	+ 1:50	-	-	-	-
538	no	no	1:25	1+1:100	1:50	1+1:100	1:100	+ 1:200	-	-	-	-
539	no	no	1:25	+ 1:100	1:25	+ 1:50	1:25	-	-	1+1:25	-	-
540	no	no	-	1+1:50	-	-	-	-	-	-	-	-

All titers negative in 1:25 dilution before first inoculation.

¹Blood samples taken and next inoculation given on same day.

²Challenge dose of 10,000 Br. abortus 2308 given 10 days after last inoculation of fraction.

³1:200 highest dilution tested.

Table 32

Titers Produced from 3 Inoculations of Fraction VII-B
and a Challenge Dose in Guinea Pigs

guinea pig number	challenged	Highest dilu- tion aggluti- nating 9 days after first inoculation ¹		Highest dilu- tion aggluti- nating 7 days after second inoculation		Highest dilu- tion aggluti- nating 10 days after third inoculation		Highest dilu- tion aggluti- nating 14 days after chal- lenging ²		Highest dilu- tion aggluti- nating 30 days after chal- lenging	
		compl.partial		compl.partial		compl.partial		compl.partial		compl.partial	
541	no	-	+ 1:25	-	+ 1:25	-	+ 1:25	-	-	-	-
542	no	-	+ 1:25	-	-	-	-	-	-	-	-
543	no	-	+ 1:25	-	-	-	-	-	-	-	-
544	no	-	1+1:25	-	+ 1:50	-	-	-	-	-	-
545	no	-	-	-	0	-	-	-	-	-	-
546	no	-	-	-	+ 1:25	-	-	-	-	-	-
547	no	1:25	+ 1:100	1:25	3+1:50	-	-	-	+ 1:25	-	-
548	no	-	+ 1:25	-	+ 1:25	-	-	-	-	-	-
549	no	-	-	-	1+1:25	-	-	-	-	-	-
550	no	1:25	1+1:100	1:25	+ 1:25	-	-	-	-	-	-
491	yes	-	-	-	-	-	-	1:100	+ 1:200	1:200 ³	-
492	yes	-	-	-	-	-	-	-	+ 1:25	1:100	2+1:200
493	yes	1:25	1+1:100	-	1+1:50	-	-	-	2+1:25	1:100	-
494	no	-	1+1:50	-	+ 1:50	dead	-	-	-	-	-
495	no	dead	-	-	-	-	-	-	-	-	-
496	yes	-	1+1:50	-	+ 1:50	-	-	1:200	-	1:200 ³	-
497	yes	-	+ 1:100	-	+ 1:50	-	-	1:50	+ 1:200	1:200 ³	-
498	yes	1:25	+ 1:100	-	+ 1:50	-	-	1:50	1+1:200	1:200 ³	-
499	yes	1:25	3+1:50	-	1+1:25	-	-	1:50	+ 1:100	1:25	+ 1:50
500	yes	-	+ 1:25	-	+ 1:25	-	-	-	-	dead	-

All titers negative in 1:25 dilution before first inoculation

¹Blood samples taken and next inoculation given on same day.

²Challenge dose of 10,000 Br. abortus 2308 given 10 days after last inoculation of fraction.

³1:200 highest dilution tested.

Table 33

Titers Produced from 3 Inoculations of Fraction V-1-C
and a Challenge Dose in Guinea Pigs

guinea pig number	challenged	infected	Highest dilu- tion agglutin- ating 10 days after first inoculation ¹		Highest dilu- tion agglutin- ating 10 days after second inoculation		Highest dilu- tion agglutin- ating 10 days after third inoculation		Highest dilu- tion agglutin- ating 14 days after chal- lenging ²		Highest dilu- tion agglutin- ating 30 days after chal- lenging	
			compl.	partial	compl.	partial	compl.	partial	compl.	partial	compl.	partial
451	no	no	-	-	-	+ 1:25	-	1+1:25	-	+ 1:25	-	-
452	no	no	-	-	-	+ 1:25	-	+ 1:25	-	-	-	-
453	no	no	-	-	-	+ 1:50	1:25	3+1:50	1:25	+ 1:100	-	-
454	no	no	-	-	-	+ 1:25	-	+ 1:25	-	+ 1:25	-	-
455	no	no	-	-	-	-	1:25	1+1:25	-	+ 1:25	-	-
456	no	no	-	-	-	-	1:25	-	-	-	-	-
457	yes	no	-	-	-	+ 1:25	-	+ 1:25	-	-	-	-
458	yes	yes	-	-	-	+ 1:25	-	-	-	-	-	+ 1:25
459	no	no	-	-	-	-	-	-	-	-	-	-
460	yes	no	-	-	-	-	-	-	-	-	-	-
461	no	no	-	-	-	-	dead	-	-	-	-	-
462	no	no	-	-	-	-	-	-	-	-	-	-
463	yes	no	-	-	-	-	-	+ 1:25	-	-	-	-
464	yes	no	-	-	-	-	1:50	1+1:100	-	-	-	-
465	yes	no	-	-	-	-	-	+ 1:50	-	+ 1:50	-	-
466	yes	yes	-	-	-	-	-	+ 1:50	1:100	+ 1:200	1:200 ³	-
467	no	no	-	-	-	-	-	-	-	-	-	-
468	yes	yes	-	-	-	-	1:25	1+1:50	-	3+1:25	1:200 ³	-
469	yes	no	-	-	-	-	1:100	-	-	+ 1:50	dead	-
470	yes	no	-	-	-	-	-	1+1:25	-	-	dead	-

All titers negative in 1:25 dilution before first inoculation

¹Blood samples taken and next inoculation given on same day.

²Challenge dose of 14,000 Br. abortus 2308 given 10 days after last inoculation of fraction.

³1:200 highest dilution tested.

Table 34

Titers Produced from 3 Inoculations of Fraction V-2-C
and a Challenge Dose in Guinea Pigs

guinea pig number	challenged	infected	Highest dilu- tion aggluti- nating 9 days after first inoculation ¹		Highest dilu- tion aggluti- nating 7 days after second inoculation		Highest dilu- tion aggluti- nating 10 days after third inoculation		Highest dilu- tion aggluti- nating 14 days after chal- lenging ²		Highest dilu- tion aggluti- nating 30 days after chal- lenging	
			compl.	partial	compl.	partial	compl.	partial	compl.	partial	compl.	partial
471	yes	no	1:25	1+1:100	1:25	+ 1:100	1:250	+ 1:500	1:100	1+1:200	-	1+1:25
472	yes	no	-	+ 1:25	1:25	1+1:50	1:200	+ 1:500	1:100	-	-	-
473	yes	yes	-	1+1:25	1:25	+ 1:100	1:250	+ 1:500	1:50	+ 1:200	1:25	+ 1:100
474	yes	no	-	+ 1:25	1:25	+ 1:100	1:250	+ 1:1000	1:50	1+1:100	-	-
475	yes	no	-	+ 1:50	1:25	+ 1:100	1:500	+ 1:1000	1:50	1+1:100	-	+ 1:25
476	yes	yes	1:25	+ 1:100	1:25	1+1:100	1:1000	+ 1:2000	1:250	+ 1:500	1:100	-
477	no	dead										
478	yes	yes	1:25	1+1:100	1:250	1+1:500	1:1000	+ 1:2000	1:250	2+1:500	1:50	3+1:100
479	yes	no	1:100	2+1:200	1:100	3+1:200	1:1000	+ 1:2000	1:200	1+1:250	1:50	+ 1:100
480	no	no	1:25	+ 1:100	1:50	+ 1:200	dead					
481	no	no	1:25	+ 1:100	1:50	1+1:100	1:25	1+1:50	1:250	-	-	+ 1:25
482	no	no	1:50	+ 1:100	1:100	+ 1:200	1:250	3+1:500	1:25	2+1:100	-	-
483	no	no	1:25	+ 1:100	1:100	+ 1:200	1:500	+ 1:2000	1:250	+ 1:500	1:100	1+1:200
484	no	no	-	-	1:100	-	1:250	+ 1:1000	1:50	3+1:100	-	+ 1:50
485	no	no	1:50	1+1:100	1:25	+ 1:50	1:500	2+1:1000	1:200	-	-	2+1:50
486	no	no	-	+ 1:100	1:25	2+1:50	dead					
487	no	no	1:50	+ 1:200	dead							
488	no	no	1:25	1+1:100	1:50	1+1:100	1:1000	-	1:200	+ 1:250	-	+ 1:25
489	yes	no	1:50	+ 1:100	1:100	2+1:200	1:1000	-	1:250	+ 1:500	-	+ 1:25
490	yes	no	1:25	1+1:50	1:50	+ 1:100	1:1000	+ 1:2000	1:200	2+1:250	1:25	+ 1:100

All titers negative in 1:25 dilution before first inoculation

¹Blood samples taken and next inoculation given on same day.

²Challenge dose of 14,000 Br. abortus 2308 given 10 days after last inoculation of fraction.

Table 35

Titers Produced from 3 Inoculations of Fraction IV-B
and a Challenge Dose in Guinea Pigs

guinea pig number	challenged	infected	Highest dilu- tion agglutin- ating 9 days after first inoculation ¹		Highest dilu- tion agglutin- ating 7 days after second inoculation		Highest dilu- tion agglutin- ating 10 days after third inoculation		Highest dilu- tion agglutin- ating 14 days after chal- lenging ²		Highest dilu- tion agglutin- ating 30 days after chal- lenging	
			compl.	partial	compl.	partial	compl.	partial	compl.	partial	compl.	partial
501	yes	yes	-	+ 1:25	-	-	-	-	1:200 ³		1:200 ³	
502	yes	yes	-	-	-	-	-	-	1:200 ³		1:200 ³	
503	yes	yes	-	-	-	-	-	-	dead			
504	yes	yes	-	-	-	-	-	-	1:200 ³		1:200 ³	
505	yes	yes	-	-	-	-	+ 1:25		1:50	+ 1:100	1:200 ³	
506	yes	yes	-	-	-	-	1:25	+ 1:100	1:100	3+1:200	1:200 ³	
507	yes	yes	-	-	-	-	-	-	1:100	+ 1:200	1:200 ³	
508	yes	yes	-	-	-	-	-	-	1:100	2+1:200	1:200 ³	
509	yes	yes	-	-	-	-	-	-	1:100	+ 1:200	1:200 ³	
510	yes	yes	-	-	-	-	-	-	1:200 ³		1:200 ³	
511	no	no	-	-	-	-	-	-	-	-	-	
512	no	no	-	-	-	-	-	-	-	-	-	
513	no	no	-	-	-	-	-	-	-	-	-	
514	no	no	-	-	-	-	-	-	-	-	-	
515	no	no	-	-	-	-	-	-	-	-	-	
516	no	no	-	-	-	-	-	-	-	-	-	
517	no	no	-	-	-	-	-	+ 1:50	-	-	-	
518	no	no	-	-	-	-	-	-	-	-	-	
519	no	no	-	-	-	-	-	-	-	-	-	
520	no	no	-	-	-	-	-	-	-	-	-	

All titers negative in 1:25 dilution before first inoculation.

¹Blood samples taken and next inoculation given on same day.

²Challenge dose of 10,000 Br. abortus 2308 given 10 days after last inoculation of fraction.

³1:200 highest dilution tested.

Table 36

Titers Produced from 3 Inoculations of Fraction II-C
and a Challenge Dose in Guinea Pigs

guinea pig number	challenged	infected	Highest dilu- tion aggluti- nating 10 days after first inoculation ¹		Highest dilu- tion aggluti- nating 10 days after second inoculation		Highest dilu- tion aggluti- nating 10 days after third inoculation		Highest dilu- tion aggluti- nating 14 days after chal- lenging ²		Highest dilu- tion aggluti- nating 30 days after chal- lenging	
			compl.	partial	compl.	partial	compl.	partial	compl.	partial	compl.	partial
411	yes	yes	1:25	1+1:50	-	+ 1:50	1:25	+ 1:50	-	-	1:100	+ 1:200
412	yes	yes	-	+ 1:100	-	3+1:25	1:25	1+1:50	-	3+1:25	1:200 ³	
413	yes	yes	1:25	1+1:50	1:50	+ 1:100	1:50	-	1:25	2+1:25	1:100	1+1:200
414	no	no	1:25	1+1:50	dead							
415	yes	no	-	-	1:25	+ 1:50	1:25	+ 1:25	-	+ 1:25	1:100	3+1:200
416	yes	no	-	-	1:25	+ 1:100	1:25	2+1:25	1:25	2+1:50	-	-
417	no	no	-	-	-	1+1:25	-	1+1:25	-	+ 1:25	-	-
418	no	no	-	-	1:50	3+1:100	1:25	+ 1:50	-	-	-	-
419	no	no	-	+ 1:25	-	+ 1:25	-	-	-	-	-	-
420	no	no	-	-	1:25	1+1:25	-	+ 1:50	-	-	-	-
421	no	no	-	-	-	-	-	-	-	-	-	-
422	no	no	-	-	dead							
423	no	no	-	-	-	-	-	+ 1:25	-	-	-	-
424	no	no	-	-	-	+ 1:25	1:25	1+1:50	-	-	-	-
425	no	no	-	-	-	-	1:25	3+1:50	-	-	-	-
426	yes	no	-	-	-	+ 1:25	-	+ 1:25	-	-	-	-
427	yes	no	-	-	-	+ 1:25	1:100	+ 1:200	dead			
428	yes	yes	-	-	-	-	-	-	-	-	1:200 ³	
429	yes	yes	-	-	-	+ 1:25	-	+ 1:25	-	-	-	-
430	yes	no	-	-	-	-	-	+ 1:25	-	-	-	-

All titers negative in 1:25 dilution before first inoculation.

¹Blood samples taken and next inoculation given on same day.

²Challenge dose of 700 Br. abortus 2308 given 10 days after last inoculation of fraction.

³1:200 highest dilution tested.

is seen that the mean titer increased after each inoculation reaching a peak of about 1:30, falling off to zero in uninfected animals 24 days after the last inoculation of fraction or 2 weeks after the challenge dose.

In figures 3 and 4 it is seen that the titer in animals which were shown to be infected by the challenge dose rose by either 2 weeks or 4 weeks while the course of both the uninfected challenged and unchallenged animals' titers tended downward in most cases. Persistent titers which had not diminished appreciably from those established at the time of challenging were seen in a few cases in animals in which no infection was established. The agglutinin titers of the infection controls were increased in all but 2 cases in which organisms were recovered by the fourth week after challenging. In the case of animals receiving the lowest challenge dose of 700 Brucella organisms only, not a single one showed a titer 2 weeks after inoculation and 2 animals failed to demonstrate a titer in spite of the recovery of organisms. Hagan (1922a) had previously found animals inoculated with very low doses of organisms to have a delayed development of titers lasting to 4 and 5 weeks.

The general picture of agglutinin production in rabbits inoculated with the fractions was the same as that in guinea pigs. Single inoculations of the fractions produced low agglutinin titers demonstrable in a week.

In vitro serological tests. It was the purpose of serological tests primarily to demonstrate the antigenic nature

of fractions. A secondary purpose was to determine if any of the fractions acting in a precipitin or absorption reaction would demonstrate strain or species specificity. Antisera for most of this work were developed in rabbits against Br. abortus 19, Br. abortus 2308, Br. suis 298. Antisera used in the earliest tests were obtained from the Army Medical School¹ and were designated Br. abortus AMS, Br. suis AMS and Br. melitensis AMS. The titers obtained when these sera were set up in two-fold dilutions against BAI antigen compared favorably with the titers found at the Army Medical School as shown in table 37 except that the titer in Br. melitensis was found to be lower, only a partial agglutination occurring in dilution of 1:2560.

Table 37

Agglutinin Titers Found in Homologous and Heterologous Sera
(AMS) Using BAI Antigen

Species	AMS titer	Titer found
<u>Br. abortus</u>	2560	2560
<u>Br. suis</u>	2560	2560
<u>Br. melitensis</u>	2560	1280

The rabbit antisera produced by the author was standardized by titration against several known homologous and heterologous strains. No great differences in reaction of a given

¹Courtesy of Capt. Arthur Abrams

strain with different sera was apparent, as shown in table 38 with one exception the BAI antigen. The agglutinating capacity of a given serum for the different strains did not vary more than one dilution in any case.

None of the sera agglutinated BAI antigen to a titer equal to the other antigens. Several factors could be responsible aside from strain difference. The other strains were viable and unwashed both on rabbit injection and agglutination. Some of the titer could have been the result of residual medium or it would seem likely some of the antigenic component was washed away from the BAI antigen.

Br. melitensis 268 serum was in insufficient quantity to test all of the antigens. In preliminary studies with AMS sera it was found that fraction I-A and fraction II-A both produced visible precipitins to about the same degree. Table 39 shows that in this case qualitative correlation existed between agglutinin and precipitin titers, Br. melitensis AMS sera being agglutinated or precipitated in lower dilution units in either case than the Br. abortus AMS or Br. suis AMS sera. The precipitin titer of Br. melitensis was proportionately lower than the agglutinin titer which could possibly indicate a lower concentration of "M" substance; however, the same mixtures centrifuged free of precipitate and set up in two fold dilutions against BAI antigen showed no agglutinins from Br. melitensis serum in 1:200 dilution while Br. abortus and Br. suis sera both showed complete agglutination in 1:200 as shown in table 40.

Table 38

**Agglutinin Titers of Brucella Species
with Heterologous and Homologous Antisera**

Antigen	Antiserum	Serum Dilutions - Final Volume														
Species	Species	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10,240	1:20,480	1:40,960	1:81,920	1:163,840	1:320,000	Antigen Only
abortus 19	abortus 19	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
	abortus 2308	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
	melitensis 268	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
	suis 298	4	4	4	4	4	4	4	4	4	4	3	+	-	-	-
abortus 128-A	abortus 19	4	4	4	4	4	4	4	4	4	1	-	-	-	-	-
	abortus 2308	4	4	4	4	4	4	4	4	4	3	-	-	-	-	-
	suis 298	4	4	4	4	4	4	4	4	4	4	4	-	-	-	-
abortus 2132	abortus 19	4	4	4	4	4	4	4	4	4	1	-	-	-	-	-
	abortus 2308	4	4	4	4	4	4	4	4	4	1	-	-	-	-	-
	suis 298	4	4	4	4	4	4	4	4	4	4	3	+	-	-	-
abortus 2308	abortus 19	4	4	4	4	4	4	4	4	4	3	-	-	-	-	-
	abortus 2308	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
	melitensis 268	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
	suis 298	4	4	4	4	4	4	4	4	4	4	4	-	-	-	-
melitensis 16m	abortus 19	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
	abortus 2308	4	4	4	4	4	4	4	4	4	1	-	-	-	-	-
	suis 298	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
melitensis 1m	abortus 19	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
	abortus 2308	4	4	4	4	4	4	4	4	4	1	-	-	-	-	-
	suis 298	4	4	4	4	4	4	4	4	4	3	-	-	-	-	-
suis 41-5	abortus 19	4	4	4	4	4	4	4	4	4	3	+	-	-	-	-
	abortus 2308	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
	suis 298	4	4	4	4	4	4	4	4	4	4	3	+	-	-	-
suis 298	abortus 19	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
	abortus 2308	4	4	4	4	4	4	4	4	4	1	-	-	-	-	-
	melitensis 268	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
	suis 298	4	4	4	4	4	4	4	4	4	4	1	-	-	-	-
suis 16303	abortus 19	4	4	4	4	4	4	4	4	4	+	-	-	-	-	-
	abortus 2308	4	4	4	4	4	4	4	4	4	2	-	-	-	-	-
	suis 298	4	4	4	4	4	4	4	4	4	4	+	-	-	-	-
BAI 1119-3 phenolized	abortus 19	4	4	4	4	4	4	4	4	-	-	-	-	-	-	-
	abortus 2308	4	4	4	4	4	4	4	2	+	-	-	-	-	-	-
	suis 298	4	4	4	4	4	4	4	4	+	-	-	-	-	-	-

Live antigens standardized to that of BAI phenolized antigen,
or 61.3 $\pm$ 0.2 per cent light transmittance using 650 milli-
microns filter

Serum dilution 0.5 ml

Antigen 0.5 ml

Incubation 48 hrs at 37 C

Later tests on precipitation with other preparations of fractions I and II confirmed the presence of precipitating antigen. However, considerable variation was experienced in the precipitin test titers. In table 41 precipitin titers of the preparations of fractions I and II are shown.

The degree of titer correlated with urease activity and absorption of agglutinating antibodies in that fraction I-A had relatively high titers, definite urease activity, and absorbed most of the agglutinating antibodies from Brucella antisera. Conversely, fraction I-B having a low titer before filtration and losing that upon filtration showed neither urease nor absorptive activity. Immunizing action to detectable degree was absent. On the other hand, fraction I-C showed definite activity on the in vitro tests and strong evidence of immunizing ability.

Fraction II preparations showed similar results except that fraction II-B did show precipitins following filtration in contrast to fraction I-B though urease activity was lost.

The D batches of preparations of fraction I showed loss of antigenicity though immediately after separation urease activity was definite. The antigenic tests were made more than a month after the fractions were isolated and at that time urease activity was absent. This fraction was not used in animals. The D batches of fraction II likewise showed a loss of urease activity though precipitin titers were definite. As shown in table 42 absorption of agglutinins was definite with this fraction but about equal in effect. Absorption with

Table 41

Precipitin Titers Produced by Preparations of
Fractions I and II with Homologous and Heterologous Sera

Preparation	Serum	Titer	
I-A	Br. abortus AMS	1:712	
I-A	Br. melitensis AMS	1:128	
I-A	Br. suis AMS	1:712	
I-B	Br. abortus 2308 (before filtration)	none 1:40	
I-C	Br. abortus 19	1:320	
I-C	Br. abortus 2308	1:320	
I-C	Br. suis 2308	1:320	
I-D ₁	Br. abortus 19	Br. abortus 19	no visible pptn.
I-D ₂	Br. abortus 2308	Br. abortus 19	" " "
I-D ₃	Br. suis 298	Br. abortus 19	" " "
I-D ₁	Br. abortus 19	Br. abortus 2308	" " "
I-D ₂	Br. abortus 2308	Br. abortus 2308	" " "
I-D ₃	Br. suis 298	Br. abortus 2308	" " "
I-D ₁	Br. abortus 19	Br. suis 298	" " "
I-D ₂	Br. abortus 2308	Br. suis 298	" " "
I-D ₃	Br. suis 298	Br. suis 298	" " "
II-A	Br. abortus AMS	1:712	
II-A	Br. melitensis AMS	1:356	
II-A	Br. suis AMS	1:712	
II-B	Br. abortus 19	1:64	
II-B	Br. abortus 2308	1:64	
II-B	Br. suis 298	1:64	
II-C	Br. abortus 19	1:640	
II-C	Br. abortus 2308	1:320	
II-C	Br. suis 298	1:640	
II-D ₁	Br. abortus 19	Br. abortus 19	320/320*
II-D ₂	Br. abortus 2308	Br. abortus 19	160/160*
II-D ₃	Br. suis 298	Br. abortus 19	80/40*
II-D ₁	Br. abortus 19	Br. abortus 2308	160/160*
II-D ₂	Br. abortus 2308	Br. abortus 2308	80/320*
II-D ₃	Br. suis 298	Br. abortus 2308	30/160*
II-D ₁	Br. abortus 19	Br. suis 298	160/320*
II-D ₂	Br. abortus 2308	Br. suis 298	160/160*
II-D ₃	Br. suis 298	Br. suis 298	320/640*

*paired runs, first on water extract; second on saline extract

Table 42

Agglutinin Absorption from Homologous and Heterologous
Strain Antisera by Fractions II-D₁, II-D₂, II-D₃

Serum			Absorption by			Titer after absorp-	
Strain	Original Titer		fraction		mg B/ml antigen*	tion	
	compl.	partial				compl.	partial
Br. abortus 19	20,480	2+1:40,960	II-D ₁	Br. abortus 19	8.4 x 10 ⁻⁴	1:200	+1:1000
Br. abortus 2308	20,480	none	II-D ₁	Br. abortus 19		1:200	3+1:2000
Br. suis 298	40,960	1+1:40,960	II-D ₁	Br. abortus 19		1:2000	none
Br. abortus 19	20,480	2+1:40,960	II-D ₂	Br. abortus 2308	8.6 x 10 ⁻⁴	1:200	1+1:2000
Br. abortus 2308	20,480	none	II-D ₂	Br. abortus 2308		1:200	+1:2000
Br. suis 298	40,960	1+1:40,960	II-D ₂	Br. abortus 2308		1:2000	none
Br. abortus 19	20,480	2+1:40,960	II-D ₃	Br. suis 298	8.4 x 10 ⁻⁴	1:200	3+1:2000
Br. abortus 2308	20,480	none	II-D ₃	Br. suis 298		1:2000	none
Br. suis 298	40,960	1+1:40,960	II-D ₃	Br. suis 298		1:200	+1:2000

Antigen - - - - 0.5 ml each tenfold dilutions 10 through 20,000

Serum - - - - - 0.5 ml of 1:10 dilution

mixed

Incubation - - 37 C water bath 4 hrs

4-6 C refrigerator 48 hrs

Precipitin test read and centrifuged

0.1 ml of supernate serially diluted in tenfold steps

2.0 ml BAI antigen added

Incubation 48 hrs

*on the basis of 1:320 dilutions of antigen

Table 43

Agglutinin Absorption from Homologous and Heterologous
Strain Antisera by Fraction II-B

Strain	Serum		Absorption by		mg N/ml antigen	Titer after absorption	
	Original titer		Fraction			complete	partial
	complete	partial					
Br. abortus 19	1:20,480	2+1:40,960	II-B	Br. abortus 2308	.012	1:20,480	+1:40,960
Br. abortus 2308	1:20,480	none	II-B	Br. abortus 2308	.012	1:10,240	+1:20,480
Br. suis 298	1:40,960	1+1:81,920	II-B	Br. abortus 2308	.012	1:40,960	none

Table 44

Agglutinin Response of Infection Control Animals

Groups I-C and II-C

guinea pig	infected	two weeks after challenge		four weeks after challenge*	
		complete	partial	complete	partial
E	yes	-	-	1:200	-
F	yes	-	-	-	-
G	no	-	-	-	-
H	yes	-	-	1:25	1+1:100
I	yes	-	-	-	1+1:25
J	yes	-	-	1:200	-
K	yes	-	-	-	3+1:25
L	yes	-	-	-	+1:50
M	yes	-	-	-	1+1:25
N	yes	-	-	-	-

*700 organisms

Groups V-1-C and V-2-C

O	yes	1:200	-	1:500	+1:1000
P	yes	1:50	+1:100	1:100	3+1:200
Q	no	-	-	-	-
R	yes	1:25	+1:50	1:100	+ 1:200
S	yes	1:25	+1:50	1:200	2+1:250

*14,000 organisms

Groups IV-B, VI-B and VII-B

T	1:50	2+1:200	1:500	+1:1000
U	1:25	+1:100	1:2000 ¹	
V	1:100	1+1:200	1:2000 ¹	
W	1:200	-	1:2000 ¹	

*10,000 organisms

¹highest dilution done

Groups V-1-B, V-2-B and VI-A

1	1:500	3+1:100
2	1:1000	1+1:2000
3	1:100	+1:200
4	1:250	+1:500
5	dead	

*7,600 organisms

Groups II-A and III-A

32	1:25	+1:50
33	1:500	-
34	1:2000	-

*8,200 organisms

fraction II-B showed dropped titers of less than one twofold dilution as shown in table 43, correlated with a titer of only 1:64 with Br. abortus or Br. suis sera, loss of urease activity, and the loss of absorptive capacity was a complete failure on the part of fraction II-B to protect guinea pigs and low degree of antibody production.

Fractions I-C and II-C showed definite precipitin activity as shown above in table 41. I-C was also tested for agglutinin absorption and absorbed nearly all of the agglutinating antibodies from 1:10 dilutions of antisera.

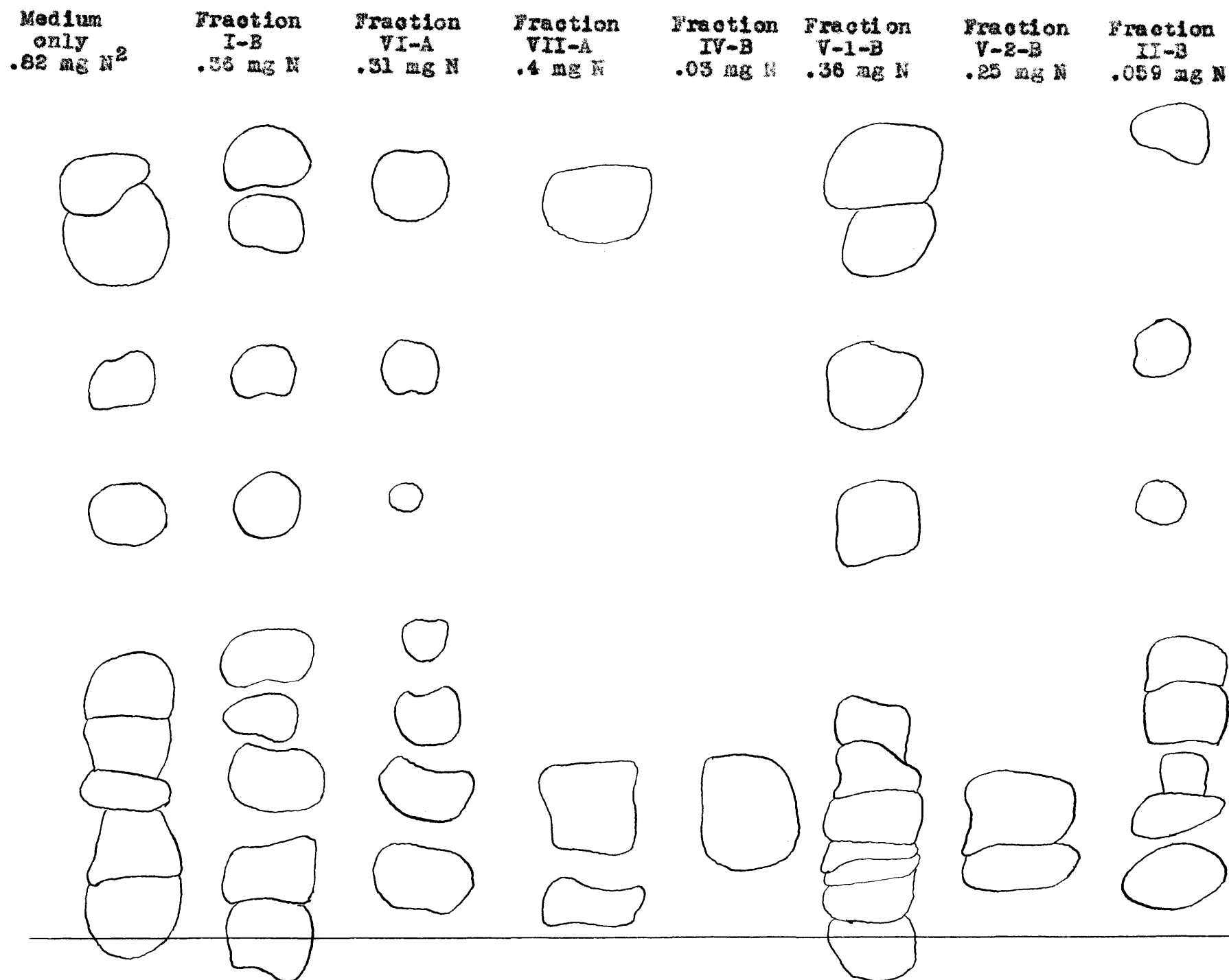
Chemical Nature of the Fractions

All but one of the fractions separated reacted strongly with biuret, ninhydrin and Molisch tests. The acetone soluble fraction IV showed a distinctly weaker biuret and ninhydrin reactivity. All fractions showed reducing sugars after hydrolysis on the basis of Benedict's test and they did not reduce with prolonged boiling with Barfoed's reagent, probably being of monosaccharide type. The peptide links and free amino groups demonstrated respectively by biuret and ninhydrin reactions coupled with antibody production as shown elsewhere in this paper suggest the protein nature of the fractions. The presence of an enzyme further indicates the likelihood of such an assumption. Inasmuch as carbohydrate was present in all of the fractions, the antigen (or agents) may contain a glycoprotein. Such agents have been found in Brucella cells by most of the workers who have isolated Brucella fractions.

The degree of purification of the given fraction is of some interest. In figure 5 the unhydrolyzed control extract of medium is seen on chromatography to have 9 ninhydrin reacting components. These could be free amino acids, peptides, or proteins. Fraction I extracted at the same pH (6.9) contains all of these but the eighth component from the bottom is seen to be diminished. The alkaline (fraction VI) and acid (fraction VII) extracts are seen to be different but several of the components from the medium are recognizable in fraction VI. Fraction II shows a reduction in number and intensity of components. Fraction IV had only a weak ninhydrin reaction and components on chromatography were difficult to distinguish. Fraction V-1 had an increase to 11 components. It should be recalled that this was a primary extract, while fraction II was a secondary extract. Fraction V-2, from the cell residue after removal of fraction V-1, showed a reduction in the number of components. Inasmuch as several amino acids are soluble in concentrated acetone it would seem that the material in Fraction IV contained few or none of these (figure 5).

Nitrogen values, done on from 1 to 4 batches of each fraction as noted on the figure, correlated with reduction of numbers of chromatographic components in fractions II, IV and V-2 would indicate some degree of purification. Decrease in the nitrogen content and components in the alkaline (VI), acid (VII), and neutral (I) aqueous extractions might indicate removal of some inert components. Methanol precipitated fraction I and II showed muchly reduced ninhydrin reactive compa-

Figure 5
Comparative Chromatograms¹ of
Unhydrolyzed Brucella Fractions



¹Developer n-butanol 48 hr. Traced from original ninhydrin positive spots.
2mg/N per ml of extract.

nents and a loss of most of the components.

Urease activity. Following the discovery in the first extractions done that urease was present in the water soluble portion it was decided to include urease tests on all of the fractions separated. It was of some interest to see if any correlations between urease activity and antigenicity and production of immunity existed.

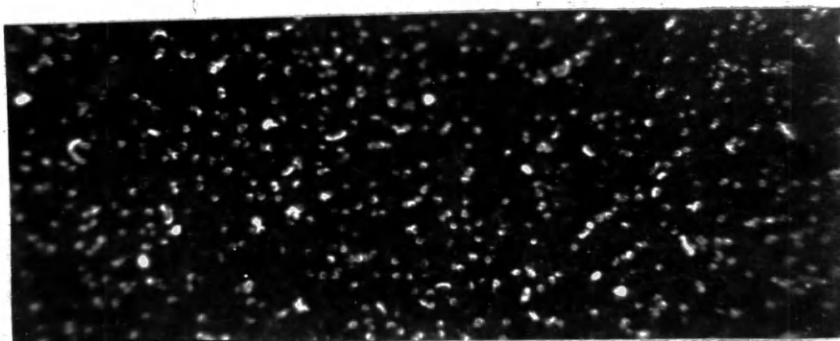
Three of the fractions studied demonstrated ability to hydrolyze urea. Heating the fraction at 90 C for 10 minutes destroyed activity. Fractions I, II and VI showed urease activity in nearly every batch, the exceptions being fractions I-B and II-B. Fraction VI was dialysed to remove base before the test was carried out. It is of interest that these fractions were both weak antigens in vitro and that none of the guinea pigs inoculated with these fractions was protected from infection. No other urease activity was noted in the fractions except the fraction IV-A in which a weak activity was noticeable on prolonged incubation in excess of 12 hours.

Results from morphological studies

A capsule could be demonstrated by india ink staining in all the species of Brucella used in this study (plate 2). Thin smears made during fractionation demonstrated an apparent diminution of the capsular size after water extraction (removal of fraction I) when compared with fresh preparations. Acetone treatment followed by saline solution (removal of fraction II) apparently removed more. However, neither these steps nor treatment of the cells with concentrated acetone (removal of

fraction IV), treatment with 30 per cent acetone alone (removal of fraction V-1) nor followed by saline (removal of fraction V-2) entirely removed the capsular material. Acid or alkaline extraction (removal of fractions VII and VI respectively) also reduced the capsular material but did not entirely remove it.

Plate 2

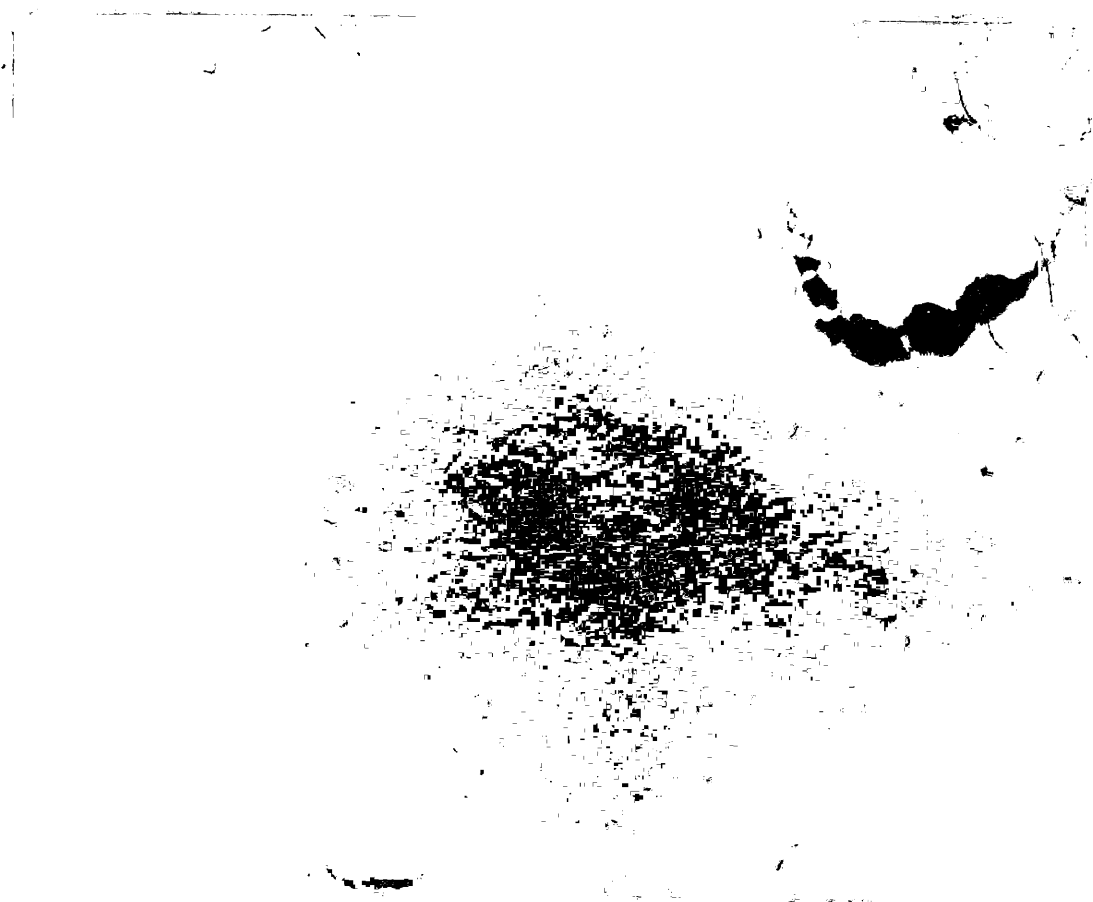


Photomicrograph of Br. abortus 2306
showing capsule 1000 X

Electron micrographs, plates 3, 4, 5 and 6, failed to show a capsule but this was probably because the cell suspensions had been killed by heating at 80 C for 15 minutes; as Huddleson (1943) points out that the capsule may be dissolved in hot water, this could be the case. At any rate, since none of several specimens examined electronoscopically demonstrated a capsule such was assumed to be the case.

Examining plates 3, 4, 5 and 6, it is seen that the water (plate 4) and alkaline (plate 5) extracted cells demonstrate the same general appearance that the fresh cell does. However, plate 3 shows 3 cells treated with concentrated acetone (fraction III-B) and plate 6 shows 3 cells so treated which stood at

Plate 3



Swelling of cell walls after acetone
extraction X 40,000

Plate 4



Cells extracted with water
X 40,000

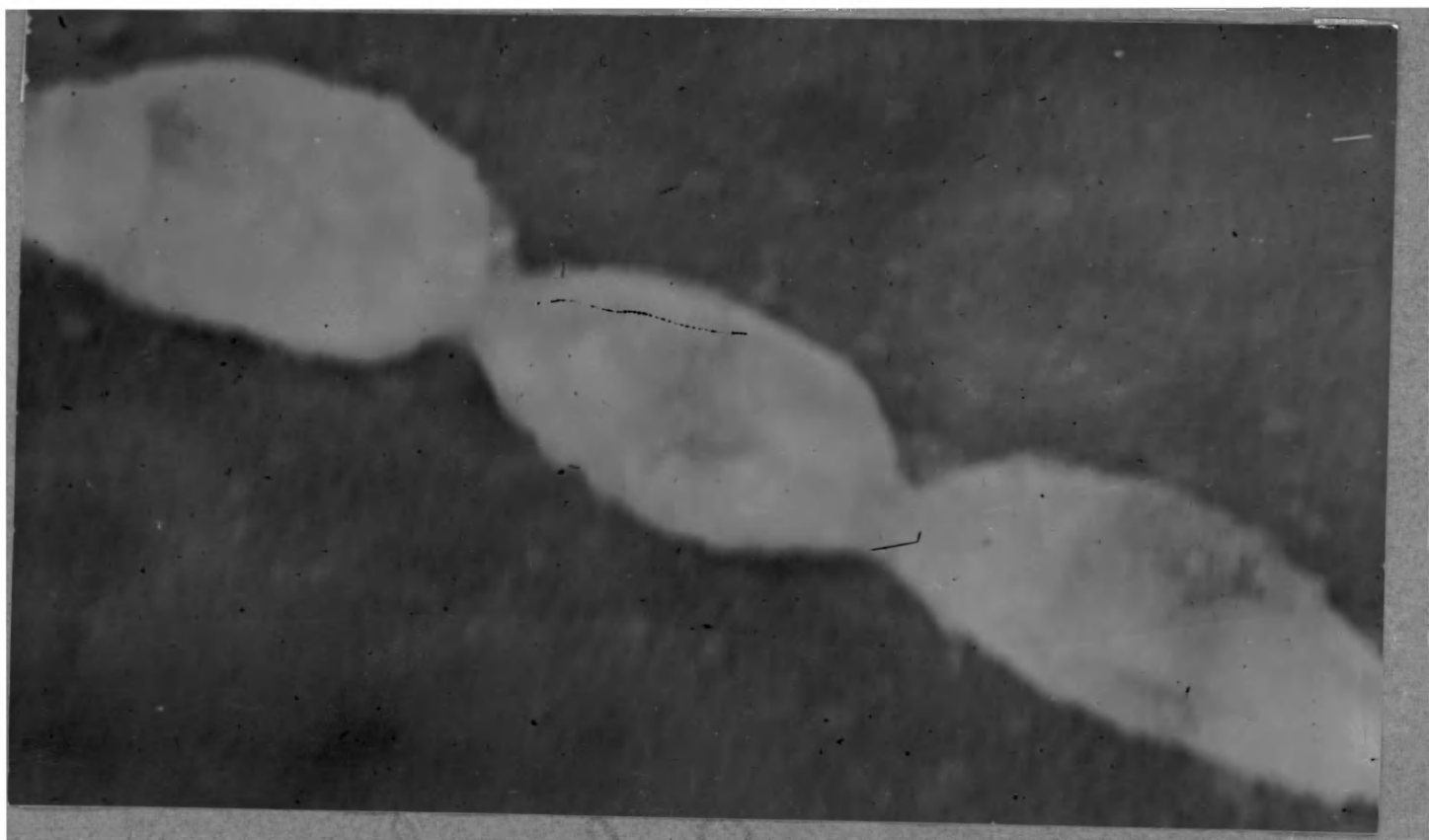


Plate 5
(left)

Cell extracted with
buffer at pH 9.2
X 120,000

Plate 6
(below)

Cells extracted with
acetone and refriger-
ated 2 weeks in water
X 84,000



4-6 C in water for 2 weeks after acetone treatment. In the former case the cell wall has apparently been removed. In plate 6 the cell wall of 2 cells seems to be swollen but not removed. In the third cell drying has caused distortion. The evidence for capsular material in the aqueous extracts being added to by cell wall material in acetone extracts seems good. Unfortunately electron micrographs of cells treated by acetone alone could not be taken. It does appear that the capsule is water soluble.

Pregnancy and abortions

Some indication of the influence of the fractions on abortions in pregnant guinea pigs was sought. In table 45 total numbers of females inoculated with each batch of fraction are presented. The number of observed abortions and living births were recorded and an effort to determine the proportion in various stages of pregnancy was made. All females were recorded as pregnant less than two weeks where doubt existed. By the third week the uterus in pregnant animals demonstrates a definite swelling over the area of the fetus and the second period was chosen from this time to 5 weeks. The 6 to 8 weeks fetus shows rather complete development including hair and this was the third period shown.

Among the challenged infection control animals were 7 females. No abortions were observed, and 4 were less than 3 weeks pregnant. Three were up to the fourth week of pregnancy and had living young. Among the unchallenged animals receiving the fractions but no challenge, of 28 females no abortions were

Table 45

Abortions Occurring in Female Guinea Pigs
Inoculated with Various Fractions and
Challenged with Br. abortus 2308

Fraction	Batch	No. of Females	Observed Abortions	Pregnancies			
				1-2 weeks	3-5 weeks	6-8 weeks	live births
I	B	5	0	3	1	0	1
	C	6	0	1	0	2	3
II	A	0	0	0	0	0	0
	B	6	0	5	1	0	0
	C	3	0	0	2	1	0
III	A	1	0	1	0	0	0
	C	6	2	4	2	0	0
IV	B	4	1	3	0	1	0
V-1	B	4	1	3	0	1	
V-1	C	7	0	4	3		
V-2	B	5	3	3	1	3 ¹	
V-2	C	7	1	2	2	1	
VI	A	4	0	3	0	1	0
	B	5		1	4	0	0
VII	B	7	1	2	2	0	0
All infec- tion con- trols		7	0	4	3	0	1
All un- challenged		28	0	3	5	12	6

¹aborted at 6 weeks

observed, 4 died early and were not counted and several females which probably were not adequately exposed to males were not pregnant. Three were in the first 2 weeks of pregnancy, 5 between the third and fifth weeks, 13 between the 6th and 8th weeks and 6 had living young. Seven females were given the viable cells of fraction III. Not a single one had a live birth, 2 aborted, and only 2 were in mid-term pregnancy at post mortem. These animals had adequate access to males, all of them being kept in contact with males for at least 7 weeks. It is important to recognize that only 4 weeks elapsed from the time that infection controls received challenge doses until they were sacrificed.

Examining the table it is seen that 7 abortions occurred among fractions IV, V-1, V-2, and VII, one of these in an uninfected but challenged animal. None were observed among the better protected animals treated with fractions I, II, and VI. No definite conclusions can be drawn but it would seem that abortions may result in guinea pigs infected with Brucella. Possibly the fractions gave some protection against abortion.

V

DISCUSSION

A summary of the animals given various batches of fraction and subsequently infected is presented in table 46. Figure 6 portrays graphically the protection or infection of all the animals inoculated with any given fraction and challenged.

From an examination of the accompanying table and figure and consideration of the individual tables presented under "Results", there seems no doubt that that protection was elicited as the result of inoculation of several of the fractions. The most successful of the fractions seems to have been the fraction extracted in alkaline aqueous suspension, fraction VI. Here all controls were infected in each of 2 tests and no animals receiving the fraction only were infected. From the table and graph it is seen that 12 of 19 animals were protected. Reduction of titers produced by inoculation of the fraction followed administration of the challenge dose, and spleen weights as shown under "Results" were somewhat lighter both in infected and uninfected challenged animals than in infection controls. Of somewhat similar nature was fraction I. However, a complete failure of I-B to protect animals on one occasion may be laid to loss of antigenic substance during filtration either through instability or absorption. When antigenic substances were present, as shown by precipitin test, and antibody production, this material was

Table 46

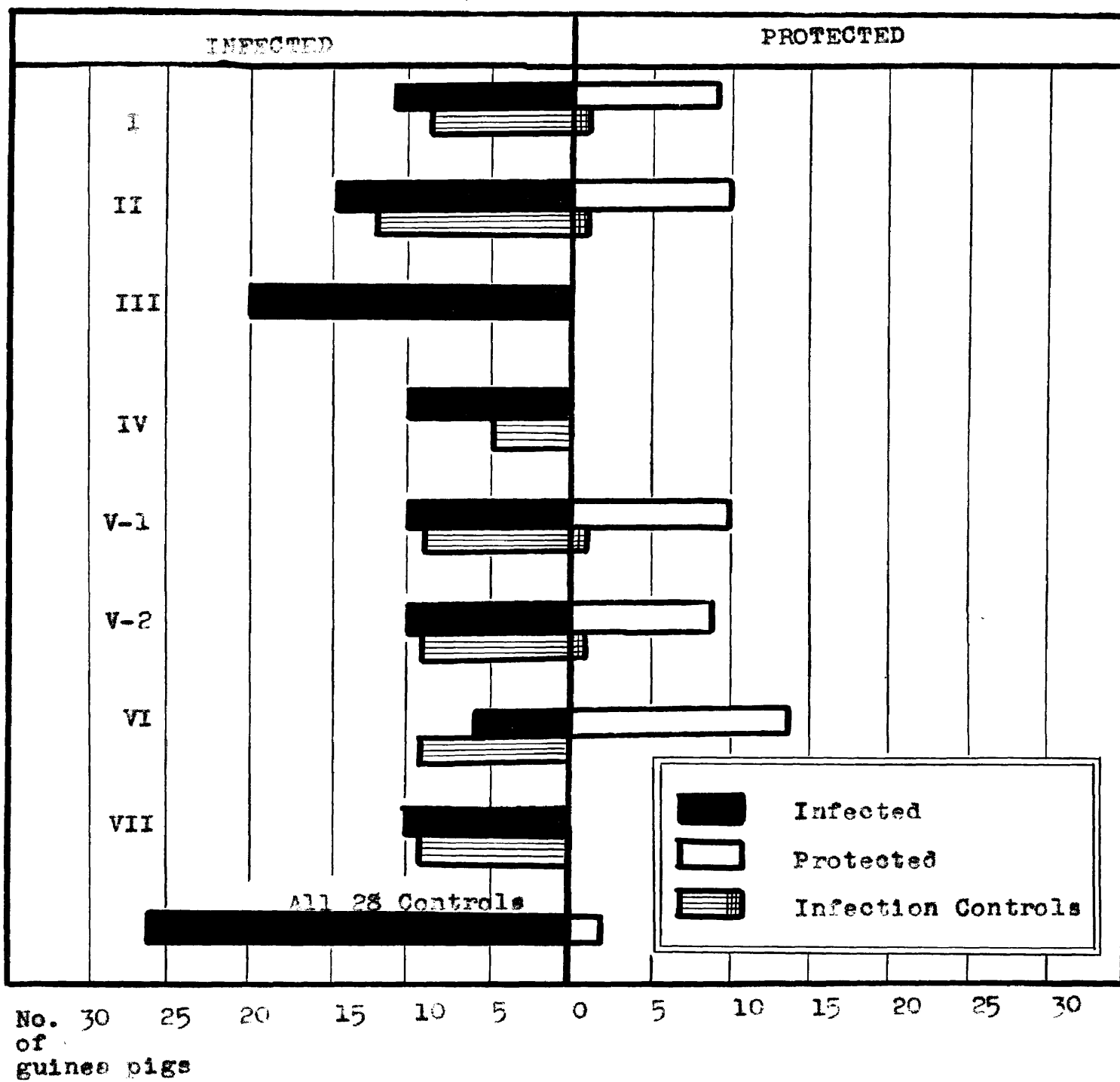
Protection of Guinea Pigs by Various Brucella Extracts

Fraction	Batch	Challenge dose	Guinea pigs used for each batch			Guinea pigs used for each fraction			
			Total no.	Challenged ¹	Infected ²	Total no.	Challenged	Infected	Uninfected
I	B	100,000	20	10	10	40	20	11	9
	C	700	20	10	1				
II	A	8,200	10	5	0	50	25	15	10
	B	100,000	20	10	10				
		700	20	10	5				
III	A	8,200	10	5	10	20	5	20	0
	C	none	10	none	10				
IV	B	10,000	20	10	10	20	10	20	0
V-1	B	7,600	19	9	6	39	19	9	10
	C	14,000	20	10	3				
V-2	B	7,600	19	9	7	39	19	10	9
	C	14,000	20	10	3				
VI	A	7,600	20	9	1	40	20	9	11
	B	10,000	20	11	6				
VII	B	10,000	20	10	10	20	10	10	0
Infection Controls 700 - 14,000			28	8	26	28	28	26	2

¹ An equal number of animals received inoculations of fractions but were not challenged, thus serving as sterility controls. None of these were infected from the above groups.

² Considered infected on basis of recovery of Brucella or titer rise following challenging.

Figure 6
Comparison of Protection
Produced by Fractions



capable of giving protection. The low challenge dose of 700 organisms may have been responsible in part though 9 of 10 animals given the challenge dose only were infected. Reference back to figure 3 shows that the alkaline extraction, fraction VI, imparted a greater stability to the immunogenic material than did extraction in neutral suspension. The difference in the antigenicity can only be ascribed to the change in pH of the medium used for extraction. Most likely a question of stability is involved.

The distressing frequency with which atypical abscesses were found on the livers in particular is ascribable to recurrent seiges of Salmonella infection. This caused most unfortunate complications in pathology and when combined with pneumonia, possibly of Streptococcus type, the pathological picture was confused. Post mortem examinations on animals from the untreated pool at various times showed the presence of patchy yellowed abscesses combined with marked inflammation of the entire small intestine and occasional splenic enlargement in cases of enteritis. Abscesses of the spleen were uncommon. Cases of pneumonia, also found in the untreated pool, usually showed marked congestion of the lung and some consolidation. A bloody mucoid pleural exudate was usually present. In such cases the spleen was usually a purplish color, thickened and pulpy, but not abscessed or nodulated on the surface. Most spleens from Brucella infected animals were distinctly nodular and frequently abscesses were visible.

Bearing these factors in mind it does not seem logical

to place great emphasis on pathology other than that following the classical description of Mabyean (1912). The spleens of infection controls given the same challenge dose were not always greater than those of infected challenged animals. Not infrequently, especially in failure of fractions I-B, II-B and I-C and II-C to immunize, tremendously enlarged spleens were found. It is known that the pathological picture increases as the disease gets older and this might suggest infection by the fraction itself except for the fact that no sterility controls were found to be infected in any case in which adequate care was taken in filtration of the fraction. The rather uncomfortable possibility that the material injected had sensitized the animal and that the exceptional enlargement of the spleen was caused by a hypersensitive reaction due to contact with fresh material is presented.

In 2 cases, guinea pigs number 27 and 411, both injected with fraction and then challenged, a pronounced edema and inflammation of the skin and a sloughing of tissue giving rise to abscesses from which Brucella were not recovered was shown following challenging. If hypersensitivity due to injection of the fraction was induced immunization would seem a risky procedure and certainly such a fraction should be more highly purified to attempt to eliminate the possibility of such a reaction.

Statistical treatment of the individual groups I-B, II-A, II-B, III, VI-A, and VII-B does not seem indicated. All, or nearly all, of the animals challenged were either infected or

protected by comparison with all 22 challenged infection controls, 20 of which were infected, or with the challenge animals used for the given group. Groups V-1-B, V-1-C, V-2-B, V-2-C and VI-B showed less marked results and the possibility of chance should be considered. Since all of 4 control animals used for group V-1-B and V-2-B were infected, the ratio of 6 infected to 9 challenged and 7 infected to 9 challenged respectively would seem to accurately indicate a certain degree of protection, though the odds are considerably decreased with the ratio of 7/9 in group V-2-C. In the case of VI-B in which 6 of 11 animals challenged were infected and all of the infection control animals were infected the odds still favor protection. However groups V-1-C and V-2-C in each of which 3 of 9 animals were infected and 1 of 5 infection controls uninfected should be examined more closely, using the method described in Topley and Wilson (1946).

vaccinated:	9	not infected:	6	or	66.7%
controls :	$\frac{5}{14}$	not infected:	1	or	$\frac{20.0\%}{46.7\%}$

$$q_0 = \frac{6}{9} + \frac{1}{5} = \frac{7}{14} = 0.5$$

$$p_0 = 1 - 0.5 = 0.5$$

then by the formula:

$$\sigma = \sqrt{p_0 q_0 \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}$$

$$\frac{0.467}{0.279} = 1.7$$

$$= \sqrt{0.5 \times 0.5 \left(\frac{1}{9} + \frac{1}{5} \right)}$$

$$\sigma = \pm 0.279$$

$$\pm \sigma = \pm 27.9\%$$

which is the standard error of differences arising by chance.

The observed difference is seen to be 1.7 times more than that of the standard error and reference to table 47 from Topley and Wilson (1946) reproduced below shows that the odds are between 6.5 and 20.98 to 1 that the animals are protected. (Not great odds but sufficient to rule out chance.)

Table 47*

Deviation in Terms of the Standard Error; or Ratio Between a Difference and its Standard Deviation	Odds against this Deviation or Difference being the Result of Random Chance
0.5	0.6 to 1
1.0	2.15 to 1
1.5	6.5 to 1
2.0	20.98 to 1
2.5	79.53 to 1
3.0	369.4 to 1
3.5	2,149 to 1
4.0	15,773 to 1
4.5	147,188 to 1

*Topley and Wilson, 1946.

Since statistically the results are usually more sound when more tests are used a look at totals of protected animals in individual groups given different preparations of the same fraction is in order. It seems justified to rule out fraction I-B at the outset since no evidence of antibody was obtained either by precipitin test or absorption from the filtered fraction. Fraction I-C with 9 of 10 vaccinated uninfected and 9 of 10 infection controls infected stands alone.

With 3 tests of fraction II 15 guinea pigs were infected out of a total of 25 guinea pigs challenged. Of a total of 13 controls 1 was not infected.

Then, from tables 44 and 46:

vaccinated:	25	not infected:	10	or 40.0%
controls :	$\frac{13}{38}$	not infected	$\frac{1}{9}$	or $\frac{7.7\%}{32.3\%}$

which by the method presented above gives:

$$\sigma = \pm .155 \text{ or } \pm 15.5 \text{ per cent standard error of difference.}$$

$$\text{ratio} = \frac{32.3}{15.5} = 2.08$$

and by table 47 the odds against the protection being by chance are nearly 20:1. The odds are rather short on the basis of the whole group. However, considering the weakened precipitin response, absorptive power, and loss of urease activity in the case of fraction II-B it may be that the immunogenic component was decreased to nearly the vanishing point. Of equal importance was the 14 fold difference in challenge dose between II-B and II-C and the use of 3 inoculations of II-C where 1 was used of II-B.

The statistical treatment of the total numbers of guinea pigs receiving fractions V-1-B and V-1-C and V-2-B and V-2-C is probably desirable. Each group showed some absence of infection with different preparations. The protection apparently increased with the 3 injections of V-1-C and V-2-C as opposed to single injections of V-1-B and V-2-B. Of 20 animals in the 2 groups inoculated with fractions V-1-B and V-1-C respectively, 9 were infected and of 19 animals inoculated in the 2 groups with fractions V-2-B and V-2-C respectively 10 were infected. In the two groups, one of 4, the other of 5, of infection controls for the 2 different groups of test animals

only 1 animal was infected. Then, from table 46:

vaccinated:	19	not infected:	10	or 52.6%
controls :	$\frac{9}{28}$	not infected:	$\frac{1}{9}$	or $\frac{11.1\%}{41.5\%}$

which by the method presented above gives:

$$\delta = \pm .186 \text{ or } \pm 18.6 \text{ per cent standard error of difference.}$$

$$\text{ratio} = \frac{41.5}{18.6} = \pm 2.22$$

the difference being ± 2.22 times the standard error the odds against the protection being by chance are more than 20:1.

A consideration of the apparent increase in protection from groups V-1-B and V-2-B to groups V-1-C and V-2-C can be made. Since distribution of uninfected animals is approximately equal a treatment of V-1-B and V-1-C should suffice. Another factor making comparison of these two groups desirable is the use of approximately equal challenge doses for groups receiving different amounts of the respective fractions. No infection controls for V-1-B and V-2-B were uninfected of 5 animals but 1 of 5 infection controls was not infected in the case of controls for V-1-C and V-2-C. Stretching the possibility of non-infected controls to 1 out of 10 on this basis the 2 groups (V-2-B and V-2-C) are compared.

For V-2-B:

vaccinated:	9	not infected:	3	or 33.4%
controls :	$\frac{9}{18}$	not infected:	$\frac{1}{2}$	or $\frac{11.1\%}{22.5\%}$

which by the method presented above gives:

$$\delta = \pm .187 \text{ or } \pm 18.7 \text{ per cent standard error of difference.}$$

The ratio $\frac{23.4}{18.7}$ is quite low (1.25) which by table 47 would

make the odds less than 5:1 that protection was due to the fractions. Protection was doubtful on this basis, though practically it should be recalled the infection controls for the first group were actually all infected and showed definite titers. For V-2-C, using data for the infection controls actually used, in which 1 was not infected:

vaccinated:	10	not infected:	7	or	70.0%
controls :	$\frac{5}{15}$	not infected:	$\frac{1}{6}$	or	$\frac{20.0\%}{50.0\%}$

which by the method presented above gives:

$\sigma = \pm .271$ or ± 27.1 per cent standard error of difference
and the ratio $\frac{50}{27.1} = 1.84$

By table 47 the odds have been increased to about 15:1. The examination of these 2 groups suggests that protection afforded by the fraction was slight. It may be that one has leaned backward in the more critical examination of these data. However, Br. abortus 2308 is a strain of considerable infectivity and Larsen (1949) and Manthei (1948) among others agree that a normal infectious dose may be as low as 10 organisms. It is further recognized that Boyd and Casman (1950) have shown that substances in tryptose agar may sometimes cause a partial inhibition of growth and experience in the author's laboratory has shown that at least one series of sub-cultures of 2308 died out on tryptose agar. Not only, then, may plate counts indicate lower numbers of organisms than are actually viable but the possibility of occasional failure to recover organisms that are present in animals must be considered in more detail.

Hagan (1922a) showed that agglutinin titers of guinea pigs

given low challenging doses of 120 to 10,000 Br. abortus occasionally were delayed as long as 4 to 5 weeks before visible agglutinins were demonstrable. In the present work the challenge doses were low throughout and infection controls were sacrificed after 30 days. The absence of titers in 2 animals receiving 700 Br. abortus with the recovery of Brucella is not, then, surprising, but the possibility that a third animal of the infection controls not showing a titer and from which organisms were not recovered was infected, exists. So too, could an occasional vaccinated animal not showing a titer harbor Brucella that were not cultured. However, the probability of missed infections, when 20 infection controls of 22 challenged were infected is not great, especially when Brucella were recovered from 9 out of 10 controls receiving sulfaguanadine. A higher challenge dose should overcome this difficulty.

VI

SUMMARY

On the basis of the results presented, the greatest degree of protection was afforded by inoculation of fraction VI. Fractions I and II showed varying degrees of protection which was probably affected by variation in the nature of preparations I-B and II-B. Protection was probably afforded from fractions V-1 and V-2 but was of a low order. Fractions IV and VII gave no evidence of protection and fraction IV produced no agglutinins in guinea pigs. The lack of response in agglutinin titers in the first groups of animals given single inoculations was probably due to the long time interval from inoculation of fraction to determination of titers.

Individual and mean agglutinin titers of groups of guinea pigs taken 7 to 10 days after each inoculation and 2 and 4 weeks after the last one clearly showed a general tendency for agglutinin titers to establish a peak and, if no more antigenic substance was given to return to zero. The tendency was general in uninfected animals with only rare exceptions. Figure 3 shows the mean agglutinin titers taken at 6 different times in the guinea pigs given 3 inoculations of fractions II-C, IV-C, V-1-C, and V-2-C. Figure 4 shows the titers produced at comparable periods by fractions I-C, VI-C, VII-C, but with a different scale of titers on the ordinate to include the higher titers produced by these fractions.

Of the acetone treated fractions tested, only V-2, the 30 per cent acetone insoluble but saline soluble residue left

after production of fraction V-1, produced detectable agglutinin titers in all guinea pigs on 3 injections. Fraction II, the saline soluble substance from cells treated with concentrated acetone, and fraction V-1 which was soluble in 30 per cent acetone and also soluble in saline, were not strong agglutinin producers. Of the 2 fractions II attained a more pronounced rise but as the mean peak titer was only 1:20 with fraction V-1 and 1:30 with fraction II the response is seen to be low. However, the biuret and Molisch tests and agglutinin response following inoculation in rabbits were evidence of at least one complete antigen which was a protein, possibly with a carbohydrate radical in most fractions. The failure of fraction IV to produce antibodies was a surprise. It had been supposed that the active portion of fraction II was acetone soluble but apparently the acetone either dissolved a carbohydrate element only, which was not capable of producing antibodies, or possibly a protein component present was denatured. The lower percentage of acetone used to dissolve fraction V-1 still did not extract a very antigenic material but subsequent treatment with saline dissolved a highly antigenic material (fraction V-2) from the cell residue left. After treatment the 30 per cent acetone was removed. It was also interesting to note that a fairly active ingredient was incompletely precipitated from fractions I and II by use of 15 per cent methanol at pH 4.7, $\gamma/2$ of .098, and -17 C. A possible relationship with the material precipitated by 30 per cent acetone at pH 5.2 exists. The distribution ratio of infected/uninfected chal-

lenged animals in groups V-1-C and V-2-C was equal in contrast to the marked difference in antigenic activity and suggests the immunogenic component was not completely precipitated by 30 per cent acetone but distributed in both precipitate and supernate.

The water soluble extracts taken at 3 different pHs all produced agglutinin titers upon injection in guinea pigs. The alkaline extract, VI-B, was the most effective of these 3 and all others and, as shown in figure 4, probably the most stable as it was capable of increasing antibody response at the time of its second inoculation. Fraction I-C produced a good initial response but apparently both it and fraction VII-B had lost antigenicity by the time the second inoculation was given. While preparations of fractions I and VI initially had urease activity fraction VII showed none. All 3 fractions showed some precipitin and absorptive activity and fractions I and VI showed some immunizing power. Fraction VII showed no immunizing capacity.

VII

CONCLUSIONS

1. There is at least one water soluble immunogenic component in the culture filtrates from Br. abortus cells which is also capable of stimulating antibody production in guinea pigs and rabbits.
2. The water soluble component is more stable in solutions of high alkalinity than in neutral or acid solutions.
3. The water soluble component is antigenic in vitro precipitating both homologous and heterologous antiserum. Marked species and strain differences are not evident.
4. The water soluble antigenic component is precipitated in part by 15 per cent methanol at -17 C from fraction I and possibly the same one is precipitated by these conditions from fraction II.
5. At least one immunogenic component withstands treatment by 30 per cent and concentrated acetone at -11 C and -60 C respectively. It is partially soluble in 30 per cent acetone, but probably insoluble in concentrated acetone at low temperatures.
6. The immunogenic component or components probably contain protein and carbohydrate. Tests for lipid were not carried out.
7. A highly antigenic component which is apparently not associated with immunization is saline soluble and precipitated by 30 per cent acetone at -11 C and pH 5.2.
8. Both water soluble components and acetone treated components are precipitinogenic in varying degrees and absorb agglutinating antisera of Br. abortus, Br. melitensis and Br. suis

Marked species and strain differences are not evident.

9. A fraction soluble in concentrated acetone only at -60 C may be haptene in nature.

10. Urease was shown to be present in the culture filtrates of Brucella. It is not known what relationship this enzyme has to immunization but its loss may be an indication of overly severe treatment of the components necessary to immunize.

VIII

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Bacto-Dextrose.....	1 g
Lead Acetate.....	0.2 g
Sodium Thiosulfate.....	0.08 g
Bacto-Agar.....	15 g
Water.....	1000 ml

F. Iron Citrate agar - Peptone Iron agar - Difco Manual (1948)

Bacto-Peptone.....	15 g
Proteose Peptone.....	5 g
Ferric Ammonium Citrate.....	0.5 g
Dipotassium Phosphate.....	1 g
Sodium Thiosulfate.....	0.08 g
Bacto-Agar.....	15 g
Water.....	1000 ml

II Dye Inhibition Tests (Huddleson 1931)(Difco 1949)

To sterile melted tryptose agar 0.1 per cent stock solutions of dye are added in sufficient quantity to make 1:100,000 dilutions of thionin, basic fuchsin, and pyronin. Steaming of the dyes for 20 minutes prior to addition of agar results in a more uniform mixture.

III Urease Solution (Anderson and Feeny 1945)

Urea.....	2.0 g
KH_2PO_4	0.1 g
K_2HPO_4 or Na_2HPO_4	0.1 g
NaCl	0.5 g
$\text{C}_2\text{H}_5\text{OH}$ (95%).....	1.0 ml
H_2O	99.0 ml

Adjust to pH 7.0 if not within 0.1 pH unit of 7.0 and add 0.5 ml 0.2 per cent aqueous solution phenol red. Sterilization is not necessary and the solution keeps well in the refrigerator. If it turns pink (alkaline) it should be discarded. Con-

trol tubes of urea solution and suspending liquid only were included in tests. For the original test 0.1 ml of a heavy cell suspension in saline is mixed with 0.1 ml of urea solution. In the work under discussion 0.2 to 0.5 ml of extract was mixed with an equal volume of urea solution. In either case incubation was in a water bath at 37 C for the time necessary to give a positive reaction. Heavy cell suspensions showed positive reaction in a matter of moments while extracts took longer. Time in excess of 24 hours was not considered. No reports were included in any tests in which the negative control tube showed any trace of pink color.

Fractions VI and VII were dialyzed and ascertained to be neutral before testing.

IV Chemical Methods

A. Nitrogen Determinations - Kabat and Mayer (1948)

To 1.0 or 2.0 ml of serum, accurately pipetted into 30 ml microkjeldahl flasks, was added 1.0 ml of concentrated sulfuric acid. For the larger volume a glass bead was added. A few grains of a mixture of equal parts of CuSO_4 and K_2SO_4 was added as catalyst and the mixture digested over an open flame on a digestion rack. Digestion was continued until the solution in the flask was clear, the flasks were cooled, a single drop of 30 per cent H_2O_2 added and the flasks heated 2 more minutes. Following digestion the flasks were cooled, stoppered, and refrigerated until titration.

For titration the solution was carefully washed into a

steam distillation apparatus using about 10 ml of water. Ten ml of a 30 per cent NaOH was next added, and the mixture distilled into a boric acid-Methyl red solution six minutes past the first change to yellow in indicator. The condenser tip of the steam distillation apparatus was immersed just under the surfact of the methyl red-boric acid solution during distillation.

The boric acid-methyl red solution was made as follows:

- A. boric acid.....40 g
water q.s. to.....1000 ml

heat to put in solution

- B. ethanol (95%)..... 60 ml
water..... 40 ml
methyl red..... 0.1 g

dissolve methyl red in water-ethanol solution.

- C. To 300 ml 4% boric acid add 10 ml solution B.

This is the indicator. It was found desirable to use glass distilled water for all reagents and in the steam generator.

Titration back to the original red color, using a control for comparison, was accomplished by titration with 0.0128 N H_2SO_4 . (Kabat and Mayer use N/70 HCl). This solution was standardized against 0.0231 N NaOH in turn standardized against acid potassium phthalate. A control of $(\text{NH}_4)_2\text{SO}_4$ included in digestion and distillation runs contained 1.00 mg N per ml of solution. It was made by placing 4.716 gm $(\text{NH}_4)_2\text{SO}_4$ in a liter volumetric flask and making to the mark with glass distilled water.

Calculation of nitrogen: Knowing the normality of the acid, the following formula was used to calculate mg nitrogen per ml of

specimen:

$$\text{mgN} = \frac{(\text{normality of H}_2\text{SO}_4 \times 14)}{(1000 \times .001)} \times \text{ccH}_2\text{SO}_4 \text{ to titrate sample}$$

14 being the atomic weight of the nitrogen of which there is 0.001 equivalent in 1000 ml of solution.

B. Tests for Carbohydrates

1. Molisch Test (Koch and Hanke 1943).

To 1.0 ml of fraction 2 drops of fresh 5 per cent alpha-naphthol were added, mixed well, and 3 ml concentrated sulfuric acid poured down the side of the tube. A layer forms and a pink to violet zone appeared at the junction when carbohydrate is present. The positive test is not wholly specific but a negative test is good evidence for absence of carbohydrates.

2. Reducing sugars before hydrolysis.

a. Benedict's solution: (Koch and Hanke 1943)

(1) Na ₂ CO ₃ (anhydr).....	100 g
Sodium citrate.....	173 g
Water to about.....	700 ml
(2) CuSO ₄	17.3 g
Water.....	100 ml

Heat and stir solution (2) until CuSO₄ is dissolved and mix with solution (1) making to final volume of 1000 ml. For use, 1 ml of fraction was added to 5 ml of reagent and heated in a boiling water bath 10 minutes. A positive reaction is indicated by a yellow to red precipitate.

V Buffers

A. Acetate buffer used in preparation of fractions V-1 and V-2:

The following were added to 250 ml cell suspension

9 ml of 1 N sodium acetate

1 ml of 1 N acetic acid

The final volume when made to 30 per cent acetone was 370 ml. Then by proportion:

$$V_1 M_1 = V_2 M_2 \quad \text{Where } V_1 \text{ and } V_2 \text{ were the first and second volumes and } M_1 \text{ and } M_2 \text{ were the first and second molarities.}$$

For sodium acetate the final molarity was:

$$9 \times 1 = 370 \times M$$

$$M = \frac{9}{370} = .0243$$

and the molarity for acetic acid was:

$$M = \frac{1}{370} = .0027$$

The degree of dissociation (α) of acetic acid was

$$\begin{aligned} \text{given by: } \alpha &= \sqrt{\frac{K}{C}} = \sqrt{\frac{1.86 \times 10^{-5}}{2.7 \times 10^{-3}}} \\ \alpha &= \sqrt{.69 \times 10^{-2}} = .083 \end{aligned}$$

The ionic concentration was given by:

$$1. \Gamma = \sum i C_i Z_i^2$$

$$2. \Gamma = (.0243 \times 1^2) + (.0243 \times 1^2) + (.0027 \times 1^2) + (.0027 \times 1^2)$$

$$\Gamma = .0486 + (.0054 \times .083)$$

$$\Gamma = .0486 + .00045 = .04905$$

It can be seen that the ionic concentration of acetic acid can be disregarded in this case.

The ionic strength may be approximated:

$$\text{and from step 2 } \Gamma/2 = \frac{.048}{2} = .024 =$$

B. Buffer used for methanol precipitation:

To 40 ml of fraction was added

1 ml 4 M sodium acetate

1 ml 10 M acetic acid

The molarities were then:

sodium acetate 0.084 M

acetic acid 0.21 M

The degree of dissociation of acetic acid was very small,
thus disregarded.

The ionic concentration was:

$$\begin{aligned}\Gamma &= \sum_i C_i Z_i^2 \\ &= (.084 \times 1^2) + (.084 \times 1^2)\end{aligned}$$

and the ionic strength was:

$$\mu = \Gamma/2 = \frac{.168}{2} = .084$$

C. Alkaline buffer used for fraction VI;

This buffer was substituted for water in extraction:

Preparation of stock buffer

M/5 H_3BO_3 and M/5 K Cl mixture - 50 ml

26.75 M/5 NaOH

dilute to 200 ml with water.

The stock buffer was further diluted to 1 liter for
use.

Then by proportion as shown above:

K Cl and H_3BO_3 were .01 M each

NaOH was .005 M

Since the degree of dissociation of boric acid was slight it was disregarded.

Then ionic concentration was calculated by:

$$\begin{aligned}\Gamma &= \sum_i C_i Z_i^2 \\ &= (.01 \times 1^2) + (.01 \times 1^2) + (.005 \times 1^2) + (.005 \times 1^2) \\ &= .02 + .01 = .03\end{aligned}$$

and approximate ionic strength is:

$$\mu \approx \Gamma/2 = \frac{.03}{2} = .015$$

D. Acid buffer used for fraction VII

This buffer was substituted for water in extraction:

Preparation of stock buffer

50.0 ml M/5 acid potassium phthalate

46.6 ml M/5 H Cl

dilute to 200 ml with water.

The stock buffer was further diluted to 1 liter for use giving the following molarities:

H Cl = .0093 M

potassium acid phthalate = .01 M

since the degree of ionization of potassium acid phthalate was small it was disregarded in calculating the ionic concentration. Then:

$$\Gamma = (.0093 \times 1^2) + (.0093 \times 1^2) \text{ and}$$

$$\mu \approx \Gamma/2 = \frac{.0093 + .0093}{2} = .019 \text{ ionic strength.}$$

VI Calculation of Mean Values

- A. Spleens: a simple arithmetic mean of animals surviving until sacrifice was made as indicated in a given group.
- B. Agglutinin titers: intermediate values were assigned on

the basis of +, 1+, 2+, or 3+ reactions in absence of complete agglutination. No partial value was used unless the next lowest dilution was at least 2+. The following table gives the values used at different dilutions of serum:

no. times diluted	Value assigned for partial agglutination			
	+	1+	2+	3+
0	5	10	15	20
25	30	35	40	45
50	60	70	80	90
100	120	140	160	180
200	210	220	230	240
250	300	350	400	450
500	600	700	800	900
1000	1200	1400	1600	1800
2000	none			