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I hereby recommend that the thesis prepared under my supervision by Paul Scott Nicholes

entitled "On the Antigenic Structure of Bacterium tularense"

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

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ON THE ANTIGENIC STRUCTURE
OF BACTERIUM TULARENSE

A dissertation submitted to the
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by

Paul Scott Nicholes

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Table of Contents

On the Antigenic Structure

of *Bacterium tularense*

The immunologic relations between *Bacterium tularense* and mammalian hosts have been studied extensively since the discovery of the organism by McCoy and Chapin (1) in 1912. Francis and Evans (1-a) studied the agglutination reaction in tularemia and established this procedure as a specific diagnostic test with more than the usual degree of reliability. Cross agglutination occurred only with organisms of the genus *Brucella*. Downs (1-b) demonstrated that the reaction between antigen and antibody fixed complement. This phenomenon has not been adequately explored; in fact, there are no published papers concerning complement fixation. Downs found that there is yet no wholly suitable antigen for use in this reaction, inasmuch as whole bacterial cells are quite anticomplementary even in fairly low concentrations. Francis (2) pointed out that one attack of tularemia imparts permanent immunity upon recovery, and that any subsequent invasion of the host by *Bact. tularense* is confined usually to a local lesion.

Foshay (3) described a diagnostic intradermal reaction by which a small amount of a light suspension of oxidized *Bact. tularense* was used as the testing agent. The test dose elicited within 48 hours an immune response at the site of injection. The test was proven to be highly specific, and responses were observed in many patients 4 days after the onset of symptoms—long before agglutinins were demonstrable in the sera. Foshay (4), (5) also described an immune response to an intradermal injection of antitularense goat serum. The transitory responses were immediate and the localized reactions appeared within 1 to 6 minutes after administration of the test dose; seldom later than the second minute.

Foshay (6), (7), (8), (9) described the beneficial use of specific antiserum in the treatment of the disease. His findings were both verified and disputed by different workers who treated patients with his and other antiserums.

Prophylaxis against the disease has also been studied. Downs (10) attempted to induce protection in rabbits by multiple injections of killed Bact. tularense. She was never able to save animals from a challenge dose of high virulence but she demonstrated increased resistance to infection by comparing the duration of life of controls and preimmunized animals after challenge.

Foshay (11) prepared chemically treated vaccines of Bact. tularense which he administered to himself over periods of several months. He postulated the production of active immunity in himself, and supported his assumption by relating that he had been exposed several times, and had at one time been subjected to an exceptionally heavy exposure without becoming infected.

Foshay, Hesselbrock, Wittenberg and Rodenberg (12) described their experience with an oxidized vaccine of Bact. tularense. They attempted immunization of some 2,145 individuals. Evidences of protection were obtained. Since then a better vaccine has been devised and found to be of greater value as a prophylactic agent.

The discussion to follow deals with a study of the chemical properties and constitution of Bact. tularense; a study which, although incomplete, contributes toward a better understanding of the immunochemistry of tularemia. The account is divided into three parts: (I) The development of methods for growing Bact. tularense in mass cultures; (II) attempts to fractionate it into antigenic and nonantigenic components, and (III) a means of quantitating the activity of certain fractions.

Historical

The literature reveals but one attempt to isolate an antigenic fraction from this organism. Foshay and Boyd (5) prepared by Ando's method a product which gave negative biuret, Millon, and ninhydrin tests, and strongly positive Molisch tests. When dissolved in physiological saline solution it precipitated strongly with antitularense serums from horses, sheep, and goats. Eleven milliliters of a solution of unknown concentration were introduced by repeated small intravenous inoculations into a yearling kid. The animal produced serum antibodies which precipitated strongly with solutions of Bact. tularense S.S.S. (Specific Soluble Substance). This serum did not agglutinate Bact. tularense, nor did it induce phagocytosis of these bacteria by leukocytes in normal citrated whole blood, but it did provoke the specific transitory skin reactions, mentioned above, in persons who had or had had the disease.

Details of further work were never published. Personal communication with Dr. Foshay disclosed that subsequent products were relatively or completely inert, and that they were mixed with substances derived from the agar medium upon which the organisms were grown. The most important of these contaminating substances was apparently a polysaccharide derived from agar-

agar, and practically inseparable from the antigenic fraction being sought.

One difficulty, then, which impeded progress was the solid medium necessary for cultivation. Only recently have Tamura and Gibby (13) and Gibby (14) first defined a liquid medium satisfactory for cultivation of this organism. With this discovery the interest in antigenic components of Bact. tularense was renewed since the possibility of isolation of substances of bacterial origin free from all other substances became assured.

Part I

Sustained rapid multiplication of organisms in test tube lots does not guarantee that the same medium will do so in bulk. This was true of the medium of Tamura and Gibby (13). Gibby (14) found it necessary to construct specially shaped culture tubes in order that, even in a minimal volume of medium, Bact. tularense would multiply rapidly. It was therefore required that the medium be adapted to mass culture techniques in order to obtain enough material for fractionation.

The first experimental mass culture adequately proved this point. A medium was prepared as follows:

1. Gelatin Hydrolyzate	2.0	per	cent
2. Cystine	0.01	"	"
3. Sodium Chloride	0.3	"	"
4. Magnesium Sulfate	0.0012	"	"
5. Potassium Phosphate (monobasic)	0.025	"	"
6. Sodium Phosphate (dibasic)	0.0123	"	"
7. Blood Cell Extract	2.0	"	"
8. Water to the volume required.			

The gelatin hydrolyzate was prepared according to the method described by Williams (16). Two per cent gelatin hydrolyzate means 2.0% in terms of whole gelatin. The hydrolyzate was a 10.0% solution of the original gelatin, hence 20 ml. were used to prepare 100 ml. of medium.

Gibby (14) first prepared blood cell extract (BCE) aseptically from rabbit blood cells. The BCE for this experiment was prepared according to his method. The 2.0% concentration used in the medium is in terms of original packed cells.

Three liters of medium were prepared in a six-liter Florence flask and sterilized at 10 pounds pressure for 20 minutes. The inoculum consisted of the entire growth from a 24 hour meat infusion dextrose cystine blood agar slant (DCBA) culture of Bact. tularense suspended in physiological saline solution. After incubation, the culture was transferred to centrifuge bottles, and the organisms were sedimented from the medium.

The results were far from satisfactory. No increase in the initial turbidity produced by the inoculum could be detected. The centrifugate was probably no greater than that which might have been harvested from the inoculum alone. The medium and the method were obviously not suitable for rapid multiplication of Bact. tularense.

Gibby (14) had observed that Bact. tularense multiplied more rapidly in a shallow than in a deep layer of liquid medium. This suggested that the oxygen demand of the organism was high, and that if the ratio of surface area to volume of medium were too low, the organism would fail to reproduce.

There were at hand some rectangular bottles of about 500 ml. capacity which, if held horizontally, presented a constant surface area of about 82.5 sq. cm. to any containable volume of medium. By varying the volume the optimal ratio of surface area to volume of medium might be determined.

The medium described above was dispensed in five bottles, each containing 10.0, 25.0, 50.0, 75.0 and 100.0 ml., respectively. The bottles were autoclaved for 15 minutes at 10 pounds pressure. Each was inoculated with 0.10, 0.25, 0.50, 0.75 and 1.00 ml., respectively, of a T-875 (M.A. 40)* suspension of a 48 hour culture of Bact. tularense in saline solution.

The cultures were incubated for 120 hours at 37° C. and the turbidities in 18 mm. tubes

* M.A. is an abbreviation for "microamperes," and represents a measurement of the electric current produced in a photoelectric cell activated by light reflected from particles in suspension. Increased turbidity of a suspension increased M.A.

were recorded in M.A. by means of a photoelectric turbidity comparator. Each culture was examined microscopically for contamination and shown to be pure. The 10 ml. culture showed but little growth, and the evaporation during the five day incubation period had left barely half of the original amount of medium. The remaining medium was highly concentrated with respect to non-volatile constituents.

The 50 ml. bottle showed by far the best growth. See table I.

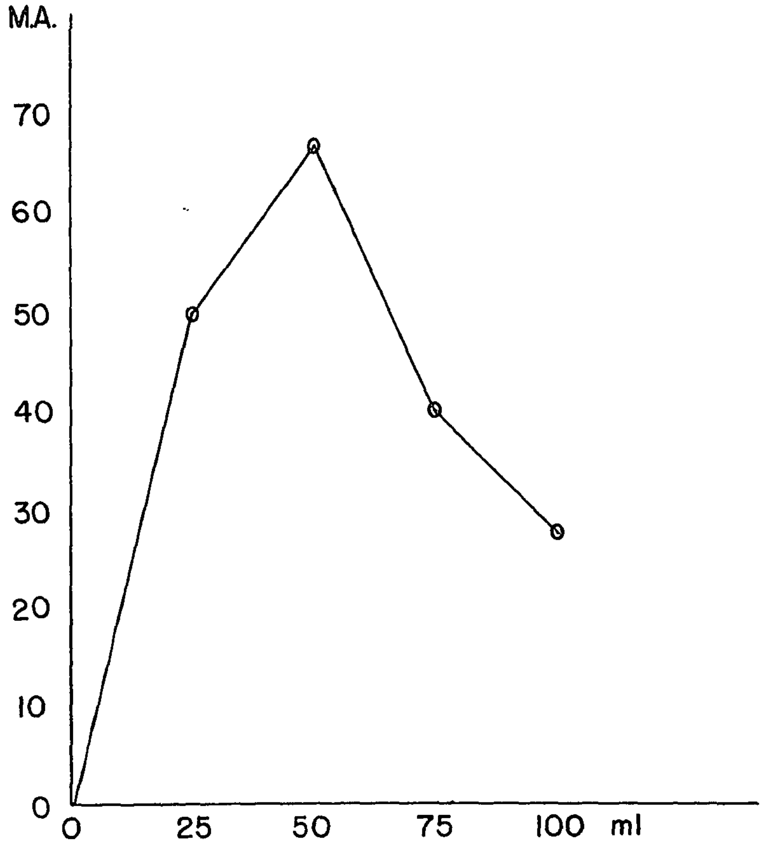
It was obvious that 50 ml. of medium was the optimal volume for these bottles. The curve (figure 1) formed by plotting microamperes (M.A.) against volume shows graphically a rise to a peak at 50 ml. of medium and a rapid drop in the amount of growth produced in the 75 ml. and 100 ml. volumes.

This experience supplied a reason for the failure of the former cultures to grow. It may be noted that the ratios of the surface area in square centimeters to the volume in milliliters in the bottles were: 8.25:1, 3.3:1, 1.65:1, 1.1:1 and 1:1.21 in the 10.0, 25.0, 50.0, 75.0 and 100.0 ml. volumes of medium, respectively. It is evident that the optimal ratio, sq. cms.:ml.) is near 1.65:1. A volume of 3000.0 ml. of medium would, at this ratio, require 4,950 sq. cm. surface area

Table I

Bottle No.	Vol. of Medium Ml.	Vol. of Inoculum Ml.	M.A. after 120 Hrs. Incubation	Ratio Sq. Cm./Vol. Ml.
1	10.0	0.10	1	8.25:1
2	25.0	0.25	50	3.30:1
3	50.0	0.50	67	1.65:1
4	75.0	0.75	40	1.10:1
5	100.0	1.00	27.5	1:1.21

Multiplication of Bacterium tularense at Various Ratios of Surface to Volume



Graphic Representation of

Table I

Figure 1

for optimal growth, whereas there were but an estimated 455 sq. cm. available in the six-liter vessel used, or a ratio of 1 sq. cm. of surface area per 10.9 ml. of medium.

Methods for growing Bact. tularense to obtain a large amount of material for study were still unsatisfactory. During a number of weeks while the method was in use the turbidities of cultures never exceeded 85 M.A. Large quantities of media necessitated the handling of too many bottles, rendering the method slow and cumbersome.

All organisms were harvested by centrifugation during these preliminary investigations. After the supernatant had been pipetted off the organisms were suspended in small amounts of salt solution and precipitated by the addition of equal volumes of acetone. A short centrifugation separated the organisms from the fluid. The supernatant was decanted, the centrifugate washed once with acetone, and removed to a small beaker. This was placed in a vacuum desiccator over anhydrous calcium sulfate and held at 10 to 15 mm. Hg pressure for 24 hours. The organisms dried to a white amorphous substance, and were stored over anhydrous calcium sulfate until a method for acetone extraction was investigated.

The evident inhibiting factor encountered in mass culture cultivation of this organism was the lack of aeration in the deep cultures. Forced aeration of the cultures would greatly facilitate the entire procedure by concentrating the culture into one vessel and, if lack of oxygen were the only impeding factor, the volume of any culture would be limited only by the size of available glass vessels, and a means of sterilizing them.

Consequently two liters of the same medium were prepared in a six-liter Florence flask. An aerator, consisting essentially of a glass tube which had a bulb at one end to contain sterile cotton for filtering the air, and which was open at the other, was inserted through the cotton stopper in the mouth of the flask. It extended to within 0.5 cm. of the bottom of the flask. The flask, medium and aerator were sterilized together in the autoclave at 10 pounds pressure for 20 minutes. The medium was inoculated with one 24 hour DCBA slant culture of Bact. tularense suspended in saline solution. Aeration was begun immediately and continued during an incubation period of 3 days.

The results, though not as good as had been expected, were encouragingly better. The possibilities of forced aeration technique for supplying the required oxygen to the organisms were further investigated.

A critical examination of the apparatus and methods of the experimental work up to this time indicated that the chief difficulties hitherto encountered were due to:

1. The bulk of the medium was too large for this type of aerator. The air emitted from the comparatively large end opening rose to the surface immediately around the conductor as separate large bubbles, and the medium in the outer circumference of the flask was disturbed very little. Aeration in this outer area was probably not extensive enough to support rapid reproduction of the organism.

2. The size of the bubbles was too large, and it was deemed probable that if the bubbles were broken up, if there were several hundred in place of one, there would be a far greater air surface presented to the liquid.

3. Although it was unknown whether agitation with aeration would contribute to or detract from optimal conditions it seemed probable that shaking the medium would give more organisms greater opportunity for contact with more molecules of gas.

Steps were taken to improve the apparatus.

The container for the medium was changed from the Florence flask to a tall, test tube shaped cylinder of approximately 2 liters capacity. Air led to the bottom of the cylinder by the conducting tube would disperse through much of the liquid during its passage from the bottom to the top.

An aerator was devised which would spray tiny bubbles into the medium in two to four directions. This aerator was essentially the same glass tube used before but the end of the tube was closed. Two to four holes of microscopic size were blown into the closed end. Air forced through these minute holes at high pressure produced myriads of minute bubbles.

Agitation of the medium in addition to this vigorous aeration was beyond control.

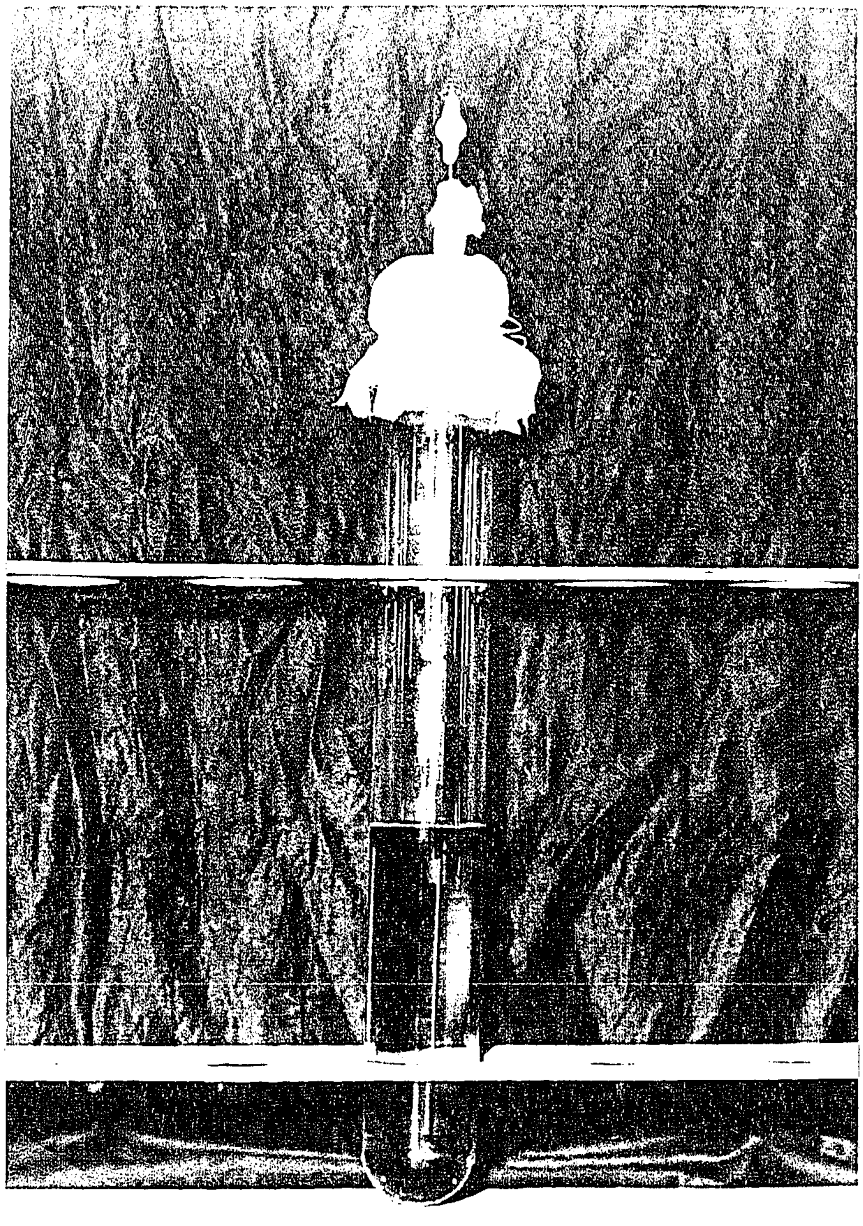
This apparatus and aeration technique were tested for several weeks. Experience soon proved that if the aerators were sterilized in the medium the tiny air outlets were often occluded. Sterilization in separate cylinders with transfer to the cultures only when aeration was to begin solved this problem. To facilitate the transfer of the aerators, and at the same time to minimize the possibility of contamination, a special plug was designed to close the mouths of the cylinders.

The closed ends of standard test tubes were cut off to form open cylinders. Two small flanges were formed on the sides of these tubes. Each tube was then rolled into the center of a cotton plug which would fit a culture cylinder, and was securely tied into position with string. The entire plug was covered with cheesecloth to avoid excessive contamination of the medium with cotton lint. The ends of the central tube were allowed to protrude approximately 1½ inches from each end of the plug. When this type of stopper was fitted into the culture cylinders the central tube furnished a small opening which could be readily flamed during transfers of inoculum and aerators. (See figure 2.)

The medium under continued study by Gibby (14), (15) had been simplified in composition as follows:

1. Gelatin Hydrolyzate	2.0	per	cent
2. Cystine	0.01	"	"
3. Glycerine	0.50	"	"
4. Sodium Chloride	0.70	"	"
5. Blood Cell Extract	10.0	"	"
6. Distilled Water to volume			

Two liters of this medium were divided in



Aeration Apparatus

Figure 2

equal portions among 3 cylinders. These were plugged with the special stoppers and sterilized at 10 pounds pressure for 20 minutes.

After cooling, the medium was inoculated with a 24 hour culture of Bact. tularense. Each cylinder received the washings of 1 meat infusion cystine glucose glycerine blood agar (DCGBA) slant culture suspended in saline solution.

After four hours' incubation the aerators were transferred aseptically to the culture cylinders and aeration begun immediately. Air was supplied by the hospital compressed air lines. Before reaching the medium the air was well washed through 2 bottles of distilled water. A trap to remove water which might have splashed into the air lines was interposed between the wash bottles and the aerators. Sterile cotton filters in the aerators served to filter out any remaining spores or bacteria.

Incubation and aeration continued for 72 hours.

Generally the results of this culture method were good, even though the degree of multiplication varied from culture to culture. Table II lists the results of 8 batches grown during 5 weeks. The M.A. readings were made in standard 18 mm. test tubes. The dry weight of the organisms

Table II

Culture No.	Amt. of Medium	Final Turbidity M.A.	Dry[2] Weights gms.	Dry Wt./2L[3] gms.
1	2 L	87	0.6401	0.6401
2	2 L	135	0.8301	0.8301
3[1]	2 L	210		
4[1]	2 L	218		
5	6 L	124	1.1240	0.3747
6	2 L	155	0.9860	0.9860
7	1.66 L	155	0.8662	1.0436
8	1.00 L	175	0.5562	1.1124

[1] Cultures were used for vaccine and were never dried. Therefore, dry weights are not given.

[2] Dry weight represents the total weight of the acetone treated and desiccated organisms grown in the amount of medium given.

[3] Equivalent dry weight equates all dry weights to dry weight per 2 liters of medium.

Growth of *Bacterium tularense* by the Aeration Method

represents the total desiccated residue of acetone extracted organisms per culture. The total dry weight of each culture batch has been equated to the dry weight per 2 liters of culture to illustrate the wide variation in yield under as nearly constant conditions of comparable inoculum, aeration, and incubation as was possible.

Obviously this method was by far the most successful yet attempted. It was employed for some time, and much of the material used in subsequent fractionation experiments was obtained by this technique.

There were some serious objections to this method. Foaming was uncontrollable. It was undesirable to have the cotton plugs saturated with moisture carried to them as foam. Ultimately several cultures became contaminated owing to this defect.

The volume of the tall vessels was not great. The foaming problem was partially controlled by reducing the volume to 2/3 liter but this necessitated more tubes for the same quantity of medium, and an equivalent increase in tubes for sterilization of the aerators. These objections added to the irregularity in culture yields stimulated further search for a better method of aeration.

Aeration of culture medium is well accomplished by shaking. Gibby (15) recorded a significant increase in growth of Bact. tularense due to the intermittent shaking incident to turbidity determinations. If readings were made every 4 hours the density of cultures increased more rapidly than if they were made every 8 hours. A series of shaken cultures was attempted in order to exploit the value of these observations. The results were so good that the shaken culture became the standard mass culture technique. A simplified and reliable mass culture technique was eventually developed. A description of this technique follows:

	per liter of medium
1. Soybean Hydrolyzate q.s. to make N	3.0 gms.
2. Cystine	0.1 gm.
3. Glycerine	5.0 ml.
4. NaCl (total)	15.0 gms.
5. Blood Cell Extract (dehydrated)	2.5 gms.
6. Distilled Water q.s.	

Sufficient hydrolyzate was added to bring the final nitrogen content to 300 mg. per 100 ml. of medium.

Fifteen grams of NaCl represents the total chloride, accomplished by adding enough NaCl to that already present in the hydrolyzate to bring the chloride content of the medium to 1.5%.

The blood cell extract was dehydrated from the frozen state by the lyophile process of Flosdorf and Mudd (25).

The soybean hydrolyzate was prepared from commercial Ortho Protein S. Ortho Protein is a soybean meal product commercially available and produced by The Drackett Company, Cincinnati, Ohio. The manufacturer described the preparation of the meal as follows:

> "Soybeans are crushed and the oil extracted from them by means of a hydrocarbon "Hexane" solvent. The resulting oil-free meal is treated with an alkaline salt solution which extracts the Ortho Protein. The alkaline solution after proper clarification is treated with an acid which precipitates out the protein in a form very similar to cottage cheese. This curd is dried and pulverized into the material of which this sample is composed."
> (Excerpt from a letter from R. A. Boyer, Director of Scientific Research, The Drackett Company.)

The hydrolyzate of this meal was at first prepared according to the method of Williams (16). It was soon observed that upon standing at refrigerator temperature, and at pH 3.0, at which pH the material had been decolorized with charcoal, a white crystalline precipitate separated from solution. The crystalline structure and the solubility characteristics of this substance suggested that it was largely tyrosine. It was soon apparent that this white crystalline material was detrimental to the growth of Bact. tularense if it was allowed to remain in the hydrolyzate. Steps were therefore taken to control precipitation, and to remove from the hydrolyzate all or most of the compound or compounds that formed the precipitate. During preparation of the hydrolyzate according to Williams' method it is concentrated twice in vacuo to a thick syrupy consistency to remove excess HCl. After each concentration the residue is restored with water to the original volume. In order to precipitate the material described above the residue was restored to ½ volume after the second concentration, the pH adjusted to 3.0, the humus cleared with charcoal, and the material placed in the cold room over night. A voluminous precipitate formed by morning. After filtration the material was restored to volume with water, decolorized a second time with charcoal, and finally concentrated in vacuo to ½ volume for storage. At refrigerator temperatures there was no further precipitation or crystallization of insoluble compounds. Media compounded with soybean hydrolyzate prepared by this method yielded abundant growth of Bact. tularense. Comparative trials with gelatin hydrolyzate and soybean hydrolyzate showed the soybean hydrolyzate to be superior for mass culture purposes.

Mass cultures were grown in 2 liter lots contained in six-liter Florence flasks. Inocula consisted of organisms washed from three or four 24 hour DCBA slants. Twenty-four hours' incubation was sufficient to reach almost maximum growth if the gases were continuously exchanged, and if the culture were continuously shaken. To accomplish an efficient exchange of gases, air was bled into the flask via a glass tube which extended through a rubber stopper at the mouth of the culture flask. The tube, fitted with a sterile cotton filter, led the incoming air to within

4 or 5 inches of the surface of the medium. The stopper was perforated at a second place and fitted with a larger glass tube plugged with cotton. This tube facilitated inoculation of the medium, and permitted the escape of gases as they accumulated over the culture. A two-liter lot yielded an average of 1.5 to 2.5 gms. of acetone dried organisms. (Figure 3.)

Table III indicates the yields that were obtained from several cultures. Most cultures were treated with phenol and converted into prophylactic vaccine. The dry weight of the yields for those cultures was, therefore, not obtained. It will be noted that there was considerable variation among yields in relation to either incubation time. This cannot be explained. Great care was exercised to ensure that media, inocula, and incubation times were uniform. Some of the irregularity may be attributed to a phenomenon noted by Gibby (15), that the light reflection properties of Bact. tularense in suspension change during continued multiplication. Often equal numbers of organisms per milliliter of medium gave widely discrepant readings on the turbidity comparator, probably due to different sizes of the preponderant number of cells among the various cultures.

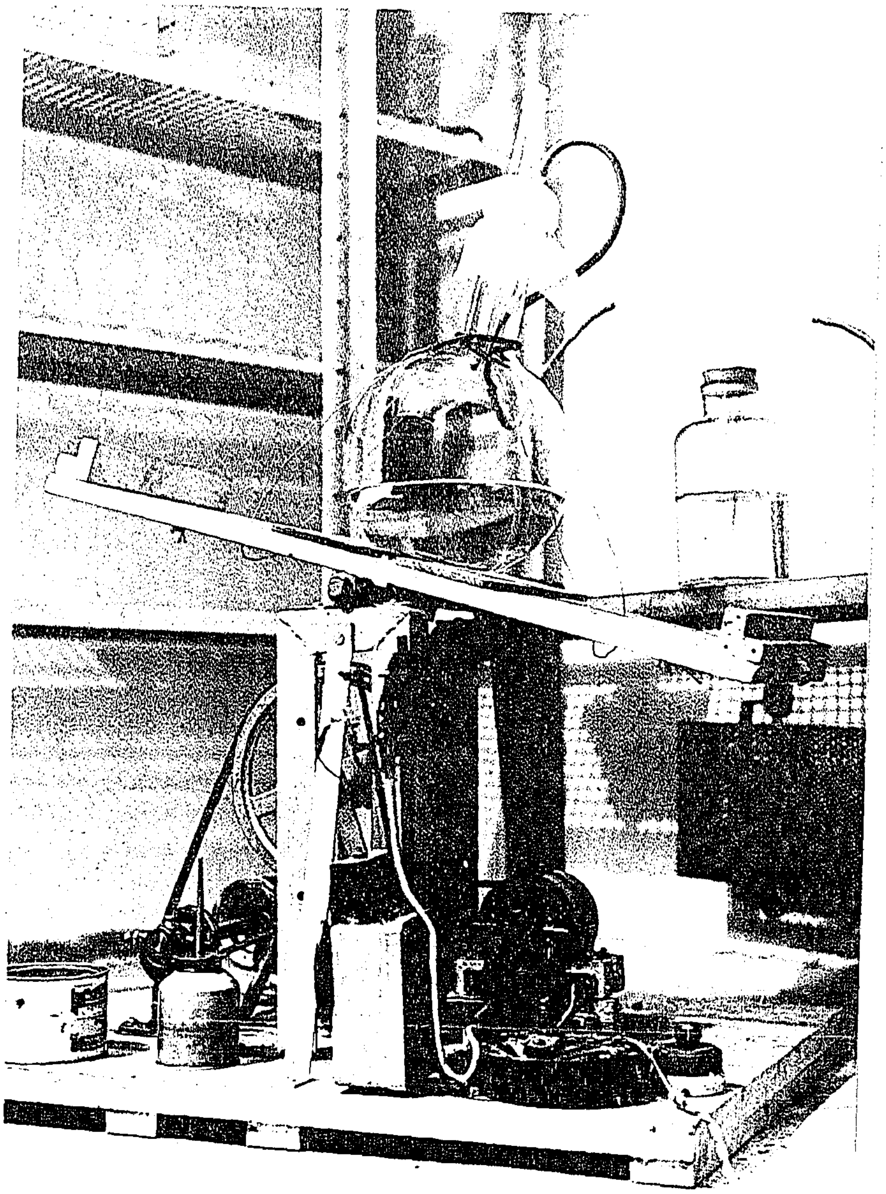
Table III

Culture No.	Incubation Hrs.	Culture Liters	M.A.	Dry Wt. Gms.
1	48	2.0	210	1.51
2	48	2.0	192	1.89
3	48	2.0	240	2.35
4	48	2.0	245	
5	24	2.0	192	
6	24	2.0	228	
7	48	2.0	204	
8	24	2.0	440	

Extent of Multiplication of

Bacterium tularense

in Shaken Culture



Shaken Culture Aeration Apparatus

Figure 3

Part II

Dochez and Avery (17) isolated from filtrates of culture media the polysaccharides that confer antigenic specificity upon pneumococcus types. Since any organism may liberate antigenic substances culture medium filtrates of Bact. tularense were investigated for the presence of soluble antigenic substances.

A sample of medium from which bacteria had been sedimented by centrifugation was filtered through a sintered glass filter. The filtrate was tested for the presence of antigenic substances as follows:

Serial dilutions of the filtrate were made in 4 mm. tubes to a final dilution of 1:15,625 in saline solution. Each dilution was 5 times greater than the preceding one. A portion of each dilution was layed over antiserum to form a sharp interface between the liquids. The tubes were incubated for 60 minutes at 37.5°C. Observations were made at 30 and at 60 minutes to discover precipitates at the interfaces. The experiment was controlled by overlaying antiserum with salt solution.

As a further test the antiserum and the

diluted filtrate in each tube were mixed by vigorous shaking, the tubes incubated for another 30 minutes, and then refrigerated over night. Observations were made the following morning.

No precipitation occurred in any dilution, and the control was also negative.

A 100 ml. portion of the filtered culture supernatant was treated with 2 volumes of cold ethyl alcohol and refrigerated over night. The flocculent precipitate was centrifuged down and the supernatant was decanted. The centrifugate was washed once with cold alcohol.

The precipitate was then taken up in water without having been allowed to dry. Much of it went into solution but after centrifugation approximately half of the original residue was thrown down as an insoluble material. That which went into solution was tested for precipitable substances as described above.

The first supernatant, and the alcohol from the subsequent washings, were pooled and the alcohol removed by distillation _in vacuo_. The remaining solution was also tested as described above.

No precipitation occurred in any dilution of either the alcohol soluble or alcohol insoluble fractions of the culture supernatant.

During investigations into the production and testing of vaccines it was observed that Bact. tularense in the soybean hydrolyzate medium was not agglutinated by homologous immune serum. This was true also for organisms which had been centrifuged from liquid cultures. However, after these organisms were washed, or after a suspension of organisms in liquid medium was dialyzed, they were readily agglutinated by immune serum.

The mechanisms of agglutination and precipitation are essentially the same. Hence it was reasoned that the precipitation procedures used might have been inadequate to prove the absence or presence of a substance in the culture supernate which would combine with antibody. Consequently the following modifications were devised to satisfy the requirements imposed by these conditions.

At the time of harvesting a 2 liter culture 50 ml. of supernatant were withdrawn and filtered through a very fine sintered glass filter to free it of particulate matter and to make it safe for free manipulation. The supernatant was placed in a Visking cellophane sac and dialyzed for 24 hours in running tap water. Thereafter the material remaining in the sac was tested for precipitable substances by the usual methods. At the time this work was done, experience had proven that the dialysis procedure was legitimate.

A faintly visible reaction at dilutions of 1:2 and 1:5 occurred after 30 minutes' incubation; after 1 hour's incubation there was no further detectable change.

Thorough mixing and refrigeration over night confirmed these observations; sediment appeared only in the 1:2 and 1:5 dilutions.

This procedure was repeated with culture supernatant after the culture had been allowed to stand at room temperature for 3 weeks to allow time for autolysis to occur if the organism produced an autolytic enzyme. The results were unchanged. Precipitates appeared at dilutions of 1:2 and 1:5.

It was concluded that very little antigenic material was formed by Bact. tularense in liquid cultures, and that an appreciable amount of autolysis detectable by this method did not occur.

Raistrick and Topley (18), Morgan (19), and Freeman, Challinor and Wilson (20) during studies of a similar nature on Eberthella typhosa, Bacterium dysenteriae, and Salmonella typhimurium respectively, first subjected the respective organisms to acetone extraction to remove substances of lipoid nature.

A pilot acetone extraction experiment was conducted on the first harvested crop of Bact. tularense to note the effect of acetone on the antigenicity of the cells in vivo.

Extraction to 2 degrees of intensity were investigated in order to approximate an optimal extraction method. A sample of acetone dried organisms was divided approximately into 2 equal parts. One was placed in a Soxhlet extractor and extracted with acetone for 24 hours. The other was placed in a centrifuge bottle with 25 to 30 ml. of acetone and incubated for 24 hours at 37°C. During this interval the bottle was shaken frequently. Upon completion of extraction the samples were dried in vacuo over anhydrous calcium sulfate at reduced pressure.

Three rabbits were chosen at random from the stock animals, numbered, and a sample of blood taken from each as a control to test for the presence of antitularense agglutinins. Each animal was treated as follows:

Rabbit No. 1 received subcutaneous injections of a suspension of cells of Bact. tularense which had been treated with acetone at 37°C. for 24 hours. The injections were administered on the first, third, and fifth days of 5 consecutive days in doses of 0.5 ml., 1.0 ml., and 1.0 ml., respectively.

Rabbit No. 2 was injected similarly with a suspension of cells which had been acetone extracted in the Soxhlet apparatus.

Rabbit No. 3 was injected similarly with a dilution of the vaccine used for immunization of human subjects against tularemia. This vaccine had been prepared by the nitrous acid oxidation method of Foshay (12), and it was used to compare its antigenicity with those of the acetone treated organisms.

The standardization of the suspensions used for this experiment was made on the turbidity comparator. As the instrument has only a limited range, and as the suspensions which were to be standardized were outside this range, it was necessary to increase the range of the instrument by putting a measured turbid suspension in the sample chamber and using its turbidity as the zero setting. The normal range of the instrument is from 0 to 200 microamperes. Ordinarily the zero point is set with distilled water in the sample chamber but it was found experimentally that the range of the machine could be increased by setting the zero with a suspension of a known turbidity and, after taking the reading of the unknown, merely adding the M.A. of the suspension which was used as zero to the M.A. given by the unknown suspension. For example: a turbid suspension reads 175 M.A. when the zero setting is made with clear distilled water in the sample chamber. With the turbid suspension in the sample chamber, the microammeter is again adjusted to zero. The unknown suspension which is greater than 200 M.A., and therefore outside the normal range, is now placed in the sample chamber, and a reading of 40 M.A. is given. Thus 175 M.A. plus 40 M.A. equals 215 M.A. This method of measuring the turbidity of suspensions greater than 200 M.A. was proven to be sufficiently accurate for this purpose.

The suspensions for injection into rabbits were standardized at 320 M.A., an arbitrary concentration with no significance other than equalization of dosage for all animals.

Fourteen days after the final injections the animals were bled and their sera subjected to agglutination tests against both formalinized Bact. tularense and the homologous antigens with which the animals had been treated.

The agglutination tests were performed by the usual macroscopic methods, and revealed that all animals had produced antibodies. The serum of animal No. 1 showed the highest titers: to 1:160 with the homologous antigen, and 1:640 with the formalinized antigen. The serum of rabbit No. 2 failed to agglutinate its homologous antigen in any dilution, but did agglutinate the formalinized antigen in dilution of 1:160. The serum of animal No. 3 agglutinated its homologous antigen in dilution of 1:10 and the formalinized antigen in dilution of 1:80. Table IV tabulates the results, and figure 4 illustrates graphically the differences in antibody titers.

Hence moderate treatment of Bact. tularense with acetone did not appreciably destroy its antigenic properties. They were still able to stimulate antibody production. Too rigorous

Table IV

Against Homologous Antigen

Serum from Rabbit No.	10	20	40	80	160	320	640	1280
1	4	4	4	3	1	0	0	0
2	0	0	0	0	0	0	0	0
3	2	0	0	0	0	0	0	0

Against Formalinized Antigen

Serum from Rabbit No.	10	20	40	80	160	320	640	1280
1	4	4	4	4	4	4	2	0
2	4	4	4	4	2	0	0	0
3	4	4	3	2	0	0	0	0

Rabbit Serum Agglutinin Titers

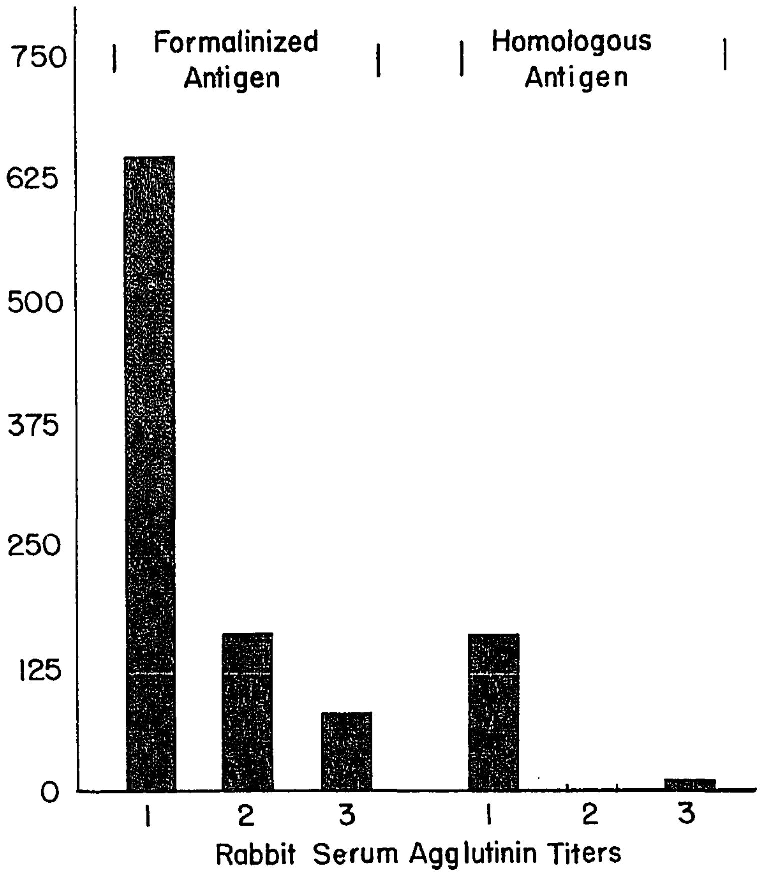

750
625
500
375
250
125
0
Formalinized Antigen
Homologous Antigen
1
2
3
1
2
3
Rabbit Serum Agglutinin Titers

Figure 4

treatment, as exemplified by the Soxhlet extraction with acetone or oxidation with nitrous acid, impaired the antigenicity of the organism.

The acetone used for the above extractions was examined for the presence of active substances that might be soluble in acetone.

All acetone used for (1) first precipitating the pooled, centrifuged organisms suspended in a minimum of physiological saline solution, (2) that used to wash the precipitated organisms, and (3) that used for the extraction was pooled and carefully filtered through paper. Two volumes of acetone were added to the filtrate to increase its concentration and to precipitate any substances soluble in lower concentrations. There was no further precipitation.

Distillation in vacuo removed the acetone and the remaining solution of salts and acetone soluble material was further concentrated to a volume of 10 to 15 ml. At no time did the distillate vapors exceed a temperature of 37°C. The concentrate was a turbid suspension which readily separated by centrifugation into water soluble and water insoluble fractions. The water soluble material was light yellow in color and quite viscous. The water insoluble material was particulate in nature and was soluble in ether, chloroform, acetone, xylene and in hot alkaline solution. It was insoluble in acid solution, and more flocculent in appearance.

The supernatant was tested for possible antigenic substances by a precipitin test against immune goat serum. The centrifugate was resuspended in saline solution and a sample was tested for activity by mixing it with various dilutions of immune serum.

Another portion of the concentrate from the distillation was dialyzed for 24 hours in running tap water and the dialyzed residue was tested similarly. No fraction showed a trace of precipitation with immune serum.

Further, a small portion of the concentrate was diluted with saline solution and 1.5 ml. were injected intravenously into a rabbit. Two injections were given at a three day interval: 0.5 ml. in the first, and 1.0 ml. on the second. The rabbit was bled 14 days thereafter, and the serum was tested for antibodies by agglutination and precipitin tests.

Again all tests were negative.

It was concluded that no antigenic material was extracted by the acetone or, if it were, its antigenicity had been destroyed.

Experience thus far showed that no antigenic substance had been isolated from the cells of *Bact. tularense* and that the acetone treated organisms were still antigenic *in vivo*. This indicated that the substances that conferred antigenic specificity on the organism were closely related to the cell body, and that in all probability the organisms would have to be broken up in order to release them.

The literature pertaining to isolation of antigenic fractions from various bacterial species presented a wide choice of methods and procedures. Five were chosen for exploratory trials: (a) the tryptic digestion method of Raistrick and Topley (18), also used by Wakeman (21) to isolate immunizing fractions from the typhoid bacillus; (b) the concentrated phenol extraction method of Palmer and Gerlough (22); (c) grinding the organisms with glass beads, a method suggested by Curran and Evans (23); (d) the diethylene glycol extraction of Morgan et al. (19); and (e) the trichloracetic acid extraction of Boivin and Mesrobeanu (24).

The Tryptic Digestion Method

Two and one-half grams of acetone dried organisms were ground to a fine powder, then suspended in 400 ml. of distilled water in a 500 ml. Erlenmeyer flask to which 0.125 gms. of trypsin (Pfahnstiel) was added, and the pH adjusted to 8.5 with 2 N NaOH. The suspension was divided equally into 500 ml. Erlenmeyer flasks, 5 ml. of toluol added to each, and incubation continued for 5 days at 37.5° C. The pH was adjusted to pH 8.5 every 24 hours, and at 60 hours another 0.05 gm. of trypsin was added to each flask. The flasks were shaken frequently to resuspend the organisms.

After incubation the contents of the flasks were combined and the toluol was removed by distillation _in vacuo_. The water bath surrounding the distilling flask never reached a temperature higher than 40°C. Distillation continued until the volume of the suspension was reduced to approximately 1/3 of its original volume.

This concentrate was centrifuged at approximately 3,000 R.P.M. for 1½ hours to remove undigested organisms and cellular debris. The supernatant was decanted. The centrifugate was resuspended in 25 ml. of distilled water, distributed into 4 conical centrifuge tubes, and spun at 4,000 R.P.M. in the angle centrifuge for 1 hour. The supernatant was decanted. The sediment was resuspended in water and again centrifuged, and the supernatant added to the former washings. The centrifugate was finally suspended in a small amount of water, washed into small tared beakers, dried at 105° C. for 24 hours, and weighed.

The supernatant from the original centrifuging was distributed into 100 ml. centrifuge flasks and centrifuged at 3,000 R.P.M. for 2 hours. A greater speed was not attempted because the centrifuge bottles tended to crush at higher speeds. The centrifugate from this operation was not great in volume. It was taken up with the washings from the main centrifugate, washed twice by centrifugation, and the washings added to the bulk of the supernatant. This fraction was designated as Di-3, a crude tryptic digest extract of *Bact. tularense.* It was an opalescent solution which precipitated readily in the presence of immune serum.

The dark field microscope showed that this crude product contained intact organisms. As antigenic activity of the extract could be attributed to the presence of these cells, their removal was desirable. The available centrifuge facilities were inadequate for a complete separation so other means were sought. It was found by pilot experiment that the Seitz filter best fitted the requirements for this separation. Therefore, the entire bulk of Di-3 was filtered through a Seitz pad. The resultant fraction received the designation Di-3F.

In vitro precipitin tests showed Di-3F to be highly reactive with immune serum, and a precipitate could be detected at an antigen dilution of 1:3,125. The immune serum had an agglutinin titer of 1:640. All the precipitin tests performed with the Di-3 series of fractions were made with the same serum, which was obtained from an immunized horse. No precipitation occurred with normal horse or human sera.

Each of 3 rabbits was injected with 2.25 ml. respectively of 1:250, 1:500, and 1:1,000 dilutions of Di-3F. Each rabbit received 0.5, 0.75, and 1.00 ml. each day on 3 successive days. The animal which received the 1:500 dilution died 2 days after the last injection. The injection material was cultured and a Gram negative rod was isolated from it. This same Gram negative rod was not isolated from the dead animal, so it cannot be assumed that this organism was the cause of death. Moreover, the other animals lived and at no time did they appear to be ill, though the Gram negative organism was present in the material given to each.

Fourteen days after the last injection the 2 survivors were bled from the heart, and the serum of each was tested for the presence of agglutinins and precipitins. Serum from bleedings made prior to the injections were tested against agglutinating antigen and the homologous antigen for the presence of agglutinins and precipitins. These tests gave the results shown in table V.

Both sera contained agglutinins and precipitins. Agglutination was observed in dilution 1:160, and precipitation of the homologous antigen occurred with a 1:25 dilution of the antigen. The latter titer was not impressive.

The tests indicated the production of antibody and one must conclude that the injected

Table V

Precipitin Titer

Dilution

1:	5	25	125	625	3,125	15,625	78,125
250	2	1	0	0	0	0	0
1,000	2	1	0	0	0	0	0
Controls	0	0	0	0	0	0	0

Agglutinin Titer

Dilution

1:	10	20	40	80	160	320	640	1280
250	4	4	4	3	2	0	0	0
1,000	4	4	4	4	2	0	0	0
Controls	0	0	0	0	0	0	0	0

Tests for Antibody Production with the Di-3F Fraction materials were antigenic. Di-3F contained substances which were insoluble in ethanol and acetone. It also gave a strongly positive Molisch test, and positive biuret, ninhydrin and Millon tests. (Table VI.) No osazones were formed when the fraction was treated with phenylhydrazine. Benedict's solution was not reduced, and hydrolysis of a sample of the fraction with strong HCl yielded no reducing substances.

A pilot experiment proved that dialysis of fraction Di-3F did not impair the activity of the fraction, and that the molecular size of the active substance was of such magnitude that it would not pass the cellophane sac. The bulk of the active fraction was therefore dialyzed for 24 hours against several changes of distilled water at 4° C. The volume ratio of dialyzed Di-3F to the dialyzate at the completion of dialysis approximated 1:30. In vacuo concentration of the dialyzate reduced the volume to approximately 400 ml. During the concentration the temperature of the distillate vapors never rose above 30°C. The concentrate manifested no activity to tularense immune serum. This material received the designation

Di-3F Dialyzate, and the dialyzed fraction the designation Di-3FD.

Di-3FD was an opalescent solution which upon prolonged centrifugation at 4,000 R.P.M. in the angle centrifuge yielded a very small amount of sediment. It gave positive ring tests with alpha-naphthol, thymol and resorcinol in the presence of sulfuric acid. The biuret and Millon's tests were negative, but a ninhydrin test gave a faintly positive reaction. (Table VI.)

Two rabbits were injected with 1.0 mg. and 0.5 mg., respectively, of Di-3FD. The material was divided into 3 equal doses and administered on 3 consecutive days. Fourteen days after the last dose the rabbits were bled and the serum of each was tested for the presence of agglutinins and precipitins. These tests were controlled with an immune serum and with serum from bleedings of the same rabbits prior to treatment with Di-3FD. The results are shown in table VII.

The tests of sera from both rabbits were entirely negative, whereas the immune controls precipitated to a titer of 1:3,125, and agglutinated formalinized <u>Bact. tularense</u> to a titer of 1:640. It must be concluded that the fraction

Table VI

	H_2O	Albumin	Di-3F	Di-3FD	Di-3FDAS$_1$	Di-3FDAI	Di-3F (Dialyzate)
Biuret	−	+	+	−	−	−	+
Ninhydrin	−	+	+	±	−	−	+
Millon's	−	+	+	−	−	−	+
Molisch	−	+	+	+	−	+	+

Chemical Tests of Fractions of the

Di-3 Series

Table VII

Precipitin Titer

	5	25	125	625	3,125	15,625
R1	0	0	0	0	0	0
R2	0	0	0	0	0	0
Positive	4	4	4	2	1	0
Negative Control	0	0	0	0	0	0

Agglutination Titer

	10	20	40	80	160	320	640	1280
R1	0	0	0	0	0	0	0	0
R2	0	0	0	0	0	0	0	0
Positive	4	4	4	4	3	2	1	0
Negative Control	0	0	0	0	0	0	0	0

R1 Serum from rabbit which received 1.0 mg. of antigen.

R2 Serum from rabbit which received 0.5 mg. of antigen.

Positive Known positive serum.

Negative Control Control sera from pre-inoculation bleedings.

Tests on Sera from Rabbits Injected with Di-3FD

was not antigenic; at least not in the concentrations administered.

It was learned by pilot experiments that a component, or components, of Di-3FD were insoluble in the organic solvents, ethanol, methanol, and acetone. Four volumes of 95% ethanol were added in the cold (4° C.) to a portion of Di-3FD. The precipitate was separated from the supernatant fluids by centrifugation, and taken up in distilled water. It dissolved rapidly. The material could be seen to "stream" into solution at the surface of the tightly packed precipitate. If the precipitate was shaken so that small clumps or masses of particles were in suspension, the fragments undergoing solution from the surface of each particle would make the particle bounce around in the liquid medium. The movement had the same appearance as Brownian movement. Prolonged centrifugation at 4,000 R.P.M. threw out of suspension only a very small amount of particulate matter which was separated from the supernatant solution by decantation. The latter was opalescent and reacted rapidly with immune serum to form a precipitate. It was given the symbol Di-3FDAI. This fraction also gave positive ring tests to alpha-naphthol, naphtho-resorcinol, menthol, thymol, and resorcinol, in the presence of sulfuric acid.

The ring colors throughout, though not exactly like those of glucose, more nearly compared with glucose than with any other sugar which was tested at the same time. Bial's test for pentose was negative. The fraction was also negative to the biuret, Millon's, and ninhydrin tests for proteins. (Table VI.)

In vivo experiments demonstrated the fraction to be devoid of antigenic properties.

The alcoholic supernatant was concentrated *in vacuo* to approximately 200 ml. Again the temperature of the concentrate was never allowed to exceed 30° C. This material was faintly opalescent in the concentrated solution, and was precipitated by tularense immune serum, but not nearly to the same degree as Di-3FDAI. This fraction was given the designation Di-3FDAS$_1$. It was non-reactive to the phenols, giving negative ring tests to those listed above. It also gave a negative test to Bial's reagent for recognition of pentose sugars, and was negative to the biuret, the Millon's and the ninhydrin tests for proteins. (Table VI.) No antibody was produced by a rabbit to which a sample of this fraction had been parenterally administered.

The alcohol insoluble fraction, Di-3FDAI was subjected to a second treatment with 4 volumes of cold 95% ethanol. It was observed that the bulk of the precipitate was not nearly as great in this second precipitation. The flocculent precipitate was centrifuged out, the supernatant decanted, and the insoluble substance dissolved in distilled water. The appearance of this solution was the same as that of the fraction Di-3FDAI except that at the same volume the opalescence appeared to be less than that of the parent product. This was considered to be the same as the former alcohol insoluble fraction and kept the same designation.

The alcoholic supernatant was concentrated *in vacuo* to remove the alcohol. The concentrate was an opalescent solution which had the same appearance in solution as that of Di-3FDAI and was readily precipitated by immune serum. This fraction received the designation Di-3FDAS$_2$. In every respect, except for its solubility in ethanol, this fraction had the same physical, chemical, and immunological characteristics of Di-3FDAI.

The bulk of the digest supernatant Di-3 was processed completely as the pilot experiments indicated. Aliquot portions of the end product fractions were dried from the frozen state by the lyophile method of Flosdorf and Mudd (25) and the quantity by weight of each fraction was calculated.

Each of the dried materials was distinctive in its appearance. The Di-3FDAI was a light gray-white fluffy material and its bulk in the drying vials was deceptive since it appeared to be the most abundant of all the fractions. However, its total weight proved to be the least. The Di-3FDAS$_1$ in the dried state was a tan colored granular material and, of all fractions, its bulk was the least for its mass. Di-3FDAS$_2$ had an appearance very much like that of Di-3FDAI. There was little difference in the bulk to mass ratio between the two. The fraction Di-3F (Dialyzate) had the appearance of crumbled flaky material and had a definite bluish tint which strongly suggested the presence of copper salts. All fractions dissolved readily in either water or physiological saline solution.

Table VIII tabulates the dry weight data from which the per cent of yield of each fraction to the total weight of the acetone treated organisms used was determined. The 1.34 gms. of material in the dialyzate which made the total weight of all fractions greater than the weight of the original organisms may be accounted

Table VIII

No.	Symbol	Description	Wt. in Gms.	Per Cent of Dry Wt. of Acetone Dried Organisms	Activity
1	Di-3FDAI	Alcohol insoluble fraction	0.14	5.6	±
2	Di-3FDAS$_1$	Alcohol soluble fraction No. 1	0.14	5.4	±
3	Di-3FDAS$_2$	Alcohol soluble fraction No. 2	0.20	4.0	±
4	Di-3F (Dialyzate)	Dialyzate of Di-3F	3.30		-
5		Residue from Digest No. 3	0.13	5.2	
6		Total weight of fractions	3.81		
7		Acetone dried organisms used for digestion	2.50		
8		Weight of un-identifiable material. No. 6 minus No. 7	1.31		
9	Di-3F (Dialyzate)	Material in dialyzate actually from the organisms, No. 4 minus No. 7	1.90	79.76	-

Fractions Obtained by Tryptic Digestion

Table IX

1:	5	25	125	625	3,125	15,625
Di-3	4	4	4	3	1	0
Di-3F	4	4	4	3	1	0
Di-3FD	4	4	4	3	1	0
Di-3FDAI	4	4	4	2	1	0
Di-3FDAS$_1$	2	1	0	0	0	0
Di-3FDAS$_2$	4	4	4	2	1	0
Di-3F (Dialyzate)	0	0	0	0	0	0
Normal Horse Serum Control	0	0	0	0	0	0

Precipitin Titers Obtained with

Certain Digest Fractions

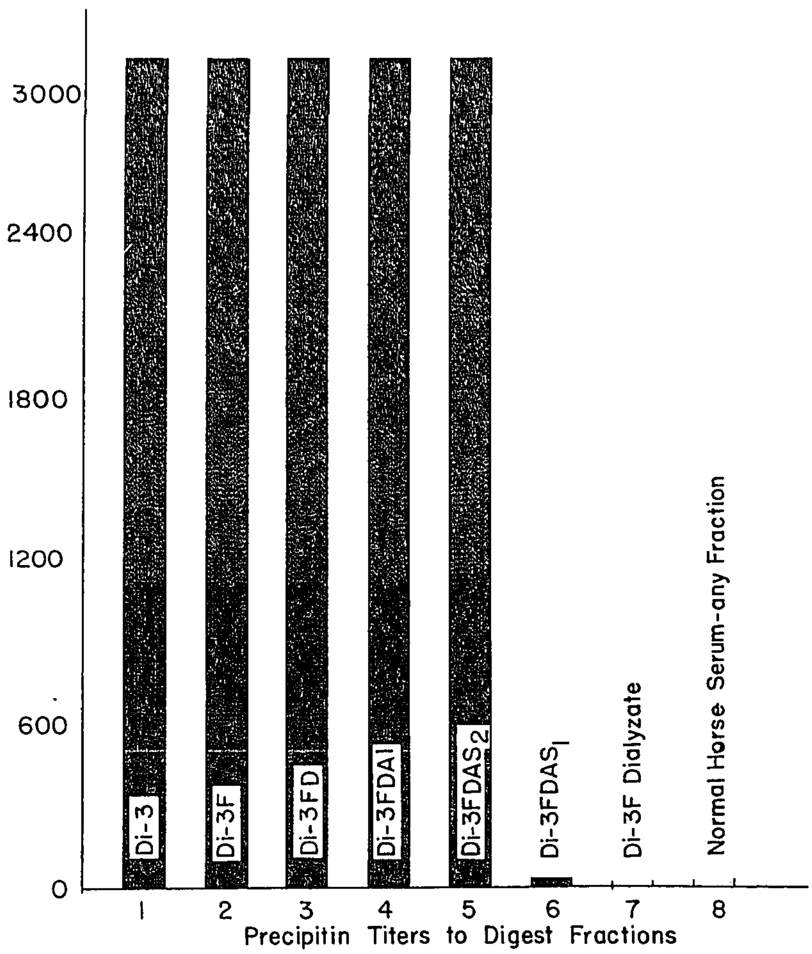

3000
2400
1800
1200
600
0
Di-3
Di-3F
Di-3FD
Di-3FDAI
Di-3FDAS2
Di-3FDAS1
Di-3F Dialyzate
Normal Horse Serum-any Fraction
1
2
3
4
5
6
7
8
Precipitin Titers to Digest Fractions

Figure 5

for by sodium hydroxide added to the digestion mixture during the digestion process. There were other contaminating substances which contributed to this excess weight, one example of which may be noted: Some copper salts were picked up from the Seitz filter which gave the filtrate a definite bluish tint and these salts were weighed with the Di-3F (Dialyzate).

The three fractions which showed antigenic activity added up to 15.0% of the total weight of the organisms used for the digestion. By far the greatest per cent, 79.76, of the original was dialyzable after digestion, and showed no activity with immune serum.

These fractions were compared quantitatively for their visible activity with immune serum. Table IX and figure 5 demonstrate the differences between the fractions. The method of quantitation was the usual serial dilution precipitin method.

The Phenol Extraction Method

Palmer and Gerlough (22) reported successful extraction of antigenic substances from typhoid bacilli by treatment with concentrated phenol. They obtained a carbohydrate fraction which was insoluble in 88-95% phenol and in alcohol, but soluble in water or in saline solution. The fraction which they isolated was actively antigenic *in vivo* as well as *in vitro*.

Two and eighty-five one-hundredths (2.85) grams of acetone dried organisms were extracted 3 times with concentrated phenol. (Enough water was added to the crystalline phenol so that at 28° C. it would remain in a liquid state. If the temperature were allowed to drop much below 28° C., it would crystallize.) A clear, almost transparent gelatinous insoluble fraction formed, was separated from the phenol by centrifugation, and washed several times with cold (4° C.) 95% ethanol to remove the phenol. The ethanol washings were preserved and pooled, the alcohol removed by distillation *in vacuo*, and the residual phenol added to the bulk of the extractant. The alcohol washed precipitate was finally washed 3 times with acetone and dried over anhydrous calcium sulfate at 14 mm. Hg pressure in a vacuum desiccator.

The resultant fraction was a white flaky and tough solid. An attempt was made to grind the substance to a powder, but that was found to be quite impossible as the flakes merely rubbed over one another, each acting as a lubricant for another rather than breaking into smaller particles. A portion of the material was put into distilled water, and shaken mechanically for several hours. As far as could be determined it was insoluble. This sample was centrifuged and the supernatant tested for visible activity in tularense immune serum but there was none. Trituration of a sample of the flaky substance effected partial solution, or more probably a suspension, of small particles. The triturated suspension gave immediate and strongly positive ring tests to alpha-naphthol, naphtha-resorcinol, and thymol in the presence of either sulfuric or hydrochloric acid, and was negative to the biuret test. Millon's test was faintly positive.

The fraction, though insoluble at neutrality, was soluble in hot hydrochloric acid, and in hot alkali. If alkali was added to the hot HCl solution, the material became insoluble at or near neutrality, and again became soluble as more alkali was added.

The triturated suspension did not reduce

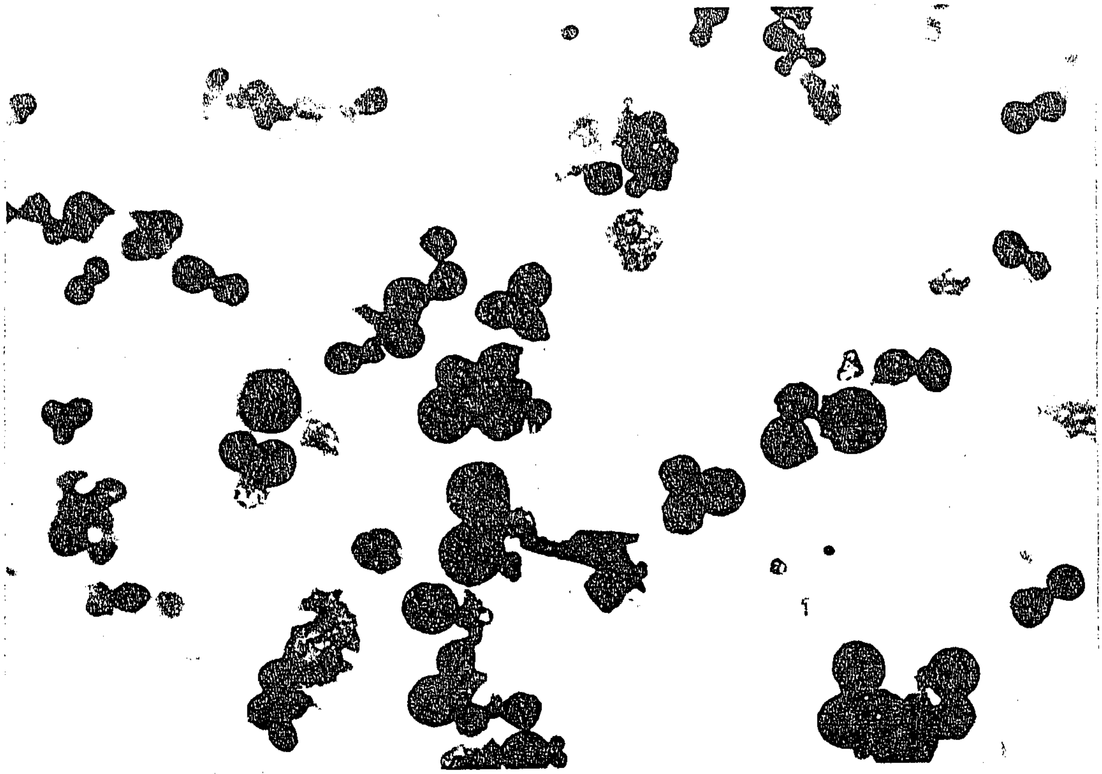
Benedict's solution, and though a solution of this fraction in HCl was heated for some time (the treatment should have initiated hydrolysis) no reducing substances were demonstrated by either Benedict's solution or methylene blue.

The fraction did not induce the production of antibody in a rabbit. Experimental evidence presented pages 54 and 55. A small portion of the triturated suspension was tested for activity against immune serum by the dilution method but no visible reaction appeared in any dilution. The substance received the designation PhI-H_2OI (Phenol insoluble, water insoluble fraction).

The entire bulk of the phenol extractant, to which had been added the residual material from the alcohol distillation, was treated with 5 volumes of distilled water. A white precipitate formed immediately. To pilot the next procedure a portion of this phenol water mixture was dialyzed through cellophane against running tap water for 18 hours. This procedure removed the phenol as well as other dialyzable substances. The material remaining in the sac was a milky suspension which, when allowed to stand for any length of time, would separate into a coarse granular sediment and an opalescent suspension.

This suspension was submitted to centrifugation for 30 minutes at 2,000 R.P.M. This treatment yielded a two layered centrifugate, the bottom layer being a granular coarse material, the top layer a creamy homogeneous material; and the dividing line was sharp between the two layers. The supernatant was very opalescent and it was readily observed that the creamy precipitate probably came from some of the material which made the solution opalescent. The very sharp line of demarcation, and the rapid settling of the granular precipitate, suggested a means of separation of the 2 centrifugates. They were thoroughly resuspended by stirring, and then centrifuged slowly (700 to 1,000 R.P.M.) in the horizontal centrifuge for 5 minutes. This time most of the coarse granular precipitate was well packed at the bottom of the centrifuge tube, and there was no creamy homogeneous material on top of the coarse centrifugate. The supernatant from this centrifuging was again spun for 5 minutes at the same slow speed. A very little additional coarse granular material separated. The supernatant was pipetted from the sediment, and the process repeated a third and a fourth time until by the last centrifugation, none of the coarse granular substance was observed to separate from the opalescent suspension. The combined sediment of the coarse granular precipitate was washed several times with small amounts of distilled water and the washings added to the opalescent supernatant. The same slow centrifuge speed was used but the time was lengthened to 15 minutes. The coarse granular fraction received the designation PhS-H_2OI (Phenol soluble, water insoluble fraction).

This fraction was never dried and cannot be described in that state. A microscopic examination of a portion stained by Gram's method showed the water insoluble material to be composed of small discrete spheres of various sizes. There was some aggregation of the spheres but never were the aggregates large. The spheres were much larger than any single Bact. tularense and they varied greatly in size (figure 6). The fraction was soluble in strongly alkaline solution. A five milli-



Fraction PhS-H_2OI

Gram Stain

Figure 6

liter sample of a heavily turbid suspension dissolved immediately upon the addition of a single drop of 30% sodium hydroxide. Twice normal sodium hydroxide was added drop by drop to a second 5 ml. sample in order to determine at which pH solution occurred. The pH of the solution, measured on the Coleman pH meter after solution was complete, was found to be 11.0. The substance was insoluble at a neutral or acid pH, and the addition of HCl caused the substance to become stringy and fibrous in appearance.

The fraction gave a positive reaction to the biuret, Millon and ninhydrin tests. The particles in the biuret test tended to settle out of suspension, and the supernatant solution was colorless. The color which accompanies a positive biuret test was on the particles.

The material was also positive to the Molisch reaction.

It did not react visibly with immune serum, nor did it stimulate production of antibody in a rabbit. (Pages 54 and 55.)

The opalescent supernatant was centrifuged at 3,000 R.P.M. for 2 hours, and the resulting centrifugate was the creamy homogeneous material described above. The supernatant was a faintly opalescent solution from which prolonged centrifugation at high speed (4,000 R.P.M.) failed to throw out any further sediment. The creamy centrifugate was collected and stored to be studied later. It was given the designation Ph-H_2OSH (Phenol water soluble heavy fraction). It was not truly water soluble but, unless it was centrifuged intensively, it would not settle from suspension. Little study was made of this material since the supernatant was of greater interest. It reacted visibly with immune serum but not nearly to the same intensity as did the opalescent supernatant. The latter was given the designation Ph-H_2OS (Phenol soluble-water soluble fraction), and it was readily precipitated by immune serum in the cold.

In view of the successful pilot fractionation the bulk of the phenol soluble material was dialyzed in running tap water for 24 hours in numerous cellophane sacs. After dialysis was complete the sacs were hung before an electric fan, and the total volume of the turbid solution was concentrated by evaporation to approximately 1/10 that of the original. The concentrate was centrifuged carefully as described above, whence the 4 fractions, Ph-H_2OI, PhS-H_2OI, Ph-H_2OSH, and Ph-H_2OS were obtained.

The fraction Ph-H_2OS, which manifested the greatest activity of all, was found to be insoluble in 3 volumes of ethanol in the cold (4°C.). The centrifugate from the first ethanol precipitation dissolved very readily in distilled water but a complete precipitation of the material a second time could not be accomplished except in the presence of sodium acetate, and at a slightly alkaline pH. Under these conditions, the precipitation of the active fraction was apparently complete. It was also noted that with each successive precipitation with alcohol there was an accompanying fall in the nitrogen content of the fraction. The product after the first precipitation yielded 3.7 to 4.7% N on analysis, and after 3 more precipitations with ethanol it contained 1.5 to 1.7% N. There was also an accompanying loss in antigenic activity inasmuch as the thrice precipitated antigen did not react with immune serum in as high a dilution as did the once precipitated antigen. The fraction also lost antigenic activity if it remained in alcohol for several days, and it developed a considerable amount of alcohol insoluble material which would not redissolve in distilled water or in saline solution. (Table X.)

After the first precipitation with ethanol an aliquot of Ph-H_2OS was dried from the frozen state by the lyophile method to compare the yield with that from the tryptic digest method. This was 16.58% of the total weight of acetone dried organisms, comparing favorably with the 15% yield obtained by tryptic digestion. The fraction was biuret, ninhydrin, and Millon negative, and strongly Molisch positive. (Table XI.)

Evidences of the presence of reducing substances in this material were unobtainable for a long time. Benedict's solution was never reduced by the fraction, even after hydrolysis in strong HCl for 4 hours. The first intimation that hydrolysis did produce a reducing substance occurred when methylene blue was used as an indicator. The pH had to be carefully controlled; only then could reduction of the indicator be noted in the presence of a portion of the hydrolyzed frac-

Table X

	1: 5	25	125	625	3,125	15,625	78,125	390,625
PhI-H_2OI	0	0	0	0	0	0	0	0
PhS-H_2OI	0	0	0	0	0	0	0	0
PH-H_2OSH	4	4	4	3	2	0	0	0
Ph-H_2OS	4	4	4	4	3	2	1	0
Ph-H_2OS (3x)*	4	4	4	4	2	1	?	0
Ph-H_2OS (3x)**	4	4	3	1	0	0	0	0
Ph-H_2OSAS	4	4	4	1	0	0	0	0

* Fraction was precipitated 3 times by ethanol in the presence of sodium acetate at an alkaline pH.

** Same as above but the last time it was precipitated it remained in the alcohol for approximately 7 days.

Precipitin Titers of Various Phenol Extraction Fractions against Tularense Immune Serum

Table XI

	PhI-H_2OI	PhS-H_2OI	Ph-H_2OSH	Ph-H_2OS	Ph-H_2OS (3x)	Ph-H_2OSAS
Biuret	-	+	-	-	-	-
Ninhydrin	-	+	-	-	-	-
Millon	+	+	-	-	-	-
Molisch	++	+	++	++	++	=

Chemical Tests of Phenol Extraction Fractions

tion. After this it was also observed that the fraction reduced methylene blue without hydrolysis. Quantitative studies with the Hagedorn and Jensen (26) technique demonstrated that a unit amount of the HCl hydrolyzed fraction reduced more potassium ferricyanide to potassium ferrocyanide than did an equivalent amount of unhydrolyzed fraction. Hydrolysis of this fraction with sodium hydroxide was unsuccessful. (Table XII.) The Ph-H_2OS fraction was never proven to be antigenic *in vivo*.

The alcohol supernatant from this series of precipitations was pooled and evaporated to a small volume in a vacuum still. The residual material was dialyzed against running tap water for 24 hours and tested for antigenic activity *in vitro*. It was precipitated by the immune serum. This fraction was given the designation Ph-H_2OS-AS (Phenol-water soluble, alcohol soluble substance).

Other extraction methods proved to be less successful. The yield of active material was low; therefore the fractionations were not carried out in detail. With further study and proper emphasis the 3 methods could probably

Table XII

Sample	Mgs. Glucose Equivalent	Per Cent Reducing Substance Glucose Equivalent
1 Unhydrolyzed Ph-H_2OS	0.058	19.3%
2 Ph-H_2OS Hydrolyzed with NaOH	0.059	19.3%
3 Ph-H_2OS Hydrolyzed with HCl	0.127	42.3%

Reducing Substance in Hydrolyzed Samples of Ph-H_2OS be made to yield good results in fractionating Bact. tularense, but with the successful fractionation obtained with the 2 methods described above, they were not used beyond a limited trial for comparative purposes.

Experimental evidence for the reaction of rabbits to certain of the fractions was found to be more conveniently presented if it were assembled. The following material explains the procedures and results of these experiments.

Felton, Sutliff, and Steele (27) showed that small amounts of type III polysaccharide induced the production of specific antibody in rabbits, whereas large amounts of the same material did not. A large and a very small dose of the Ph-H_2OS fraction was tested for its property of stimulating antibody production in rabbits.

Rabbits received doses of Ph-H_2OS as indicated below.

No.	Amt. injected in mg.	Route	Dosage/day in mg.		
			1	2	3
118	5.0	IV	1.0	2.0	2.0
113	5.0	SQ	1.0	2.0	2.0
105	0.05	IV	0.01	0.02	0.02
112	0.05	SQ	0.01	0.02	0.02

IV indicates intravenous route of injection.

SQ indicates subcutaneous route of injection.

A preinoculation bleeding was performed on each rabbit, and the serum of each was tested for the presence of agglutinins and precipitins.

Sixteen days following the last injection all rabbits were bled from the heart and the serum of each was tested for agglutinins and precipitins. The tests were controlled with a known positive immune serum.

None of the rabbits produced detectable antibody. Hence the Ph-H_2OS portion was not antigenic *in vivo*. Table XIII shows the observed findings.

The antigenic effect in rabbits of the fractions, Ph-H_2OS, PhI-H_2OI, PhS-H_2OI, and the acetone soluble fraction from the acetone extraction procedure, individually and collectively, was not known.

Rabbits received doses of antigens as indicated below.

No.	Fraction	Route	Dosage/day in ml. 1	3
4	Acetone Soluble	IV	0.5	1.0
5	PhS-H_2OI	SQ	0.5	1.0
6	Ph-H_2OS	IV	0.5	1.0
7	PhI-H_2OI	IV	0.5	1.0

Table XIII

	Animal No.	Precipitin Titers							Agglutinin Titers							
		5	25	125	625	3,125	15,625	78,125	10	20	40	80	160	320	640	1280
Preinoculation Titers	118	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	113	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	105	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	112	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Control	4	4	4	4	3	2	1	4	4	4	4	4	3	2	0
Postinoculation Titers	118	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	113	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	105	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	112	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Control	4	4	4	4	3	2	1	4	4	4	4	4	3	2	0

Precipitation and Agglutination Tests of Rabbit Sera

IV indicates intravenous route of injection.

SQ indicates subcutaneous route of injection.

The concentrations of the various test substances were not measured since only the possible activities as antigens were to be tested. Before the test substances were administered the rabbits were bled from the heart and the serum of each was tested for the presence of agglutinins and precipitins. Fifteen days after the last dose the animals were again bled and the serum of each was tested similarly. Controls of known positive serum were performed with the unknowns. No serum except the positive control reacted with the antigen. Table XIV shows the observed data.

It was thought that possibly these 4 substances might be antigenic if they were administered as a mixture. Rabbit No. 5 was subjected to subcutaneous injections of a mixture of all 4 antigens during a period of 7 days, receiving a total of 4.0 ml. of the mixture. Fourteen days after the last dose was administered the rabbit was again bled and its serum was tested for precipitins and agglutinins.

Precipitation tests of the serum of this animal were negative in all dilutions as were

Table XIV

		Animal No.	Precipitin Titers 5	25	125	625	3,125	15,625	78,125	Agglutinin Titers 10	20	40	80	160	320	640	1280
Preinoculation Titers	PhS-H_2OI	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	PhI-H_2OI	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Ph-H_2OS	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Acetone Sol.	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Control	4	4	4	4	3	2	1	4	4	4	4	4	3	2	0
Postinoculation Titers	PhS-H_2OI	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	PhI-H_2OI	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Ph-H_2OS	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Acetone Sol.	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Control	4	4	4	4	3	2	1	4	4	4	4	4	3	2	0

Precipitation and Agglutination Tests of Rabbit Sera agglutination tests against formalinized *Bact. tularense*.

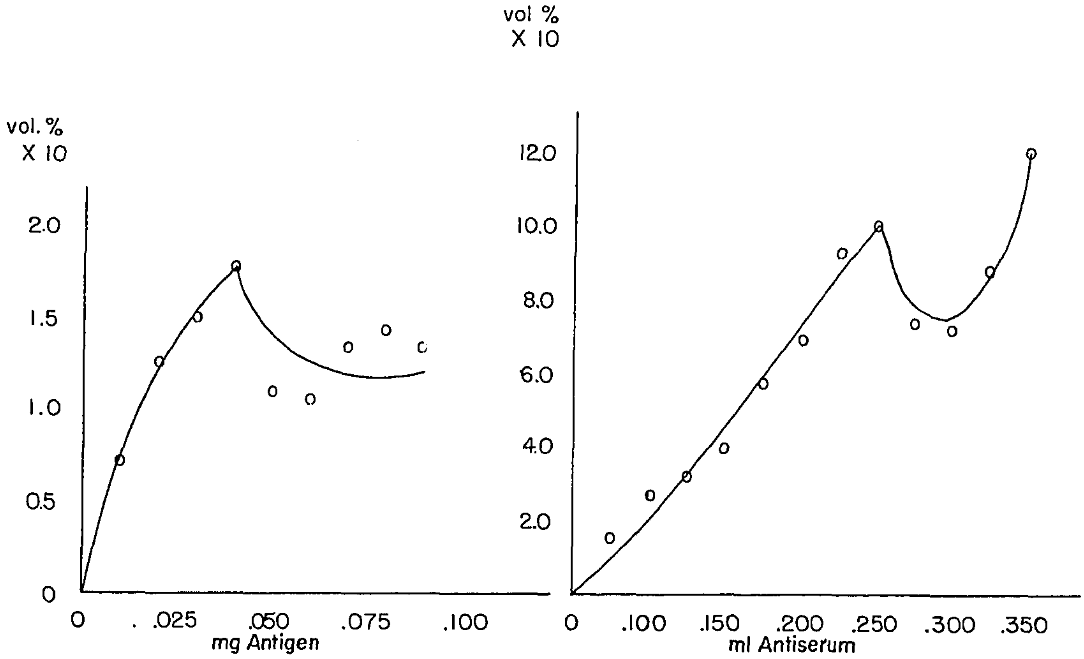
Therefore the fractions isolated from *Bact. tularense* by phenol extraction were not antigenic *in vivo*, either singly or collectively.

Part III

During the course of the work the need became apparent for a method that was capable of detecting small differences in the activity of various antigens. The serial dilution method used to compare the activity of the antigens was not considered adequate for the detection of small differences of activity. Boyd (28) warns that if each successive dilution doubles the previous dilution in any system, the error in the determination is automatically 50%, and that 100% error is possible since it is difficult to choose which of 2 tubes shows the final dilution wherein a visible reaction has occurred. The error is even greater if the dilutions are fivefold or tenfold. Thus it follows that if 2 different antigens were capable of reacting with antiserum to the same dilution they are not necessarily of the same potency per unit of mass of antigen. If of course there were differences in dilutions to which a reaction could be observed the difference was valid, and the one antigen could be assumed to have a greater activity.

Gibby (15) had prepared some modified hematocrit tubes to compare the culture yields of Bact. tularense. These tubes measured the packed volumes of cells and, by simple calculation, the relative proportion of cells to volume was determinable. The method was very successful and it was hoped that the same technique would measure the volumes of a series of packed specific precipitates. Ascending amounts of one reactant were added to a constant amount of the other, and the volume made constant by the addition of saline solution. After incubation for 2 hours the precipitate in each tube was well suspended and the proportions of precipitates to total volumes were determined. Theoretically the greatest relative volume of precipitate should have been found in the tube in which the proportion of antigen to antibody was at or near the equivalence point, where there was no excess of either or a slight excess of both.

Table XV and figure 7 illustrate that the procedure did not produce the expected results. These data are representative of a number of similar trials. The curves were always smooth and regular until they reached a certain proportion of antigen to antibody, then they broke and wandered. This occurred



Volume per cent of Precipitates

Figure 7

Table XV

Mg. Antigen	Vol. % x 10
0.00	0.00
0.01	0.72
0.02	1.26
0.03	1.50
0.04	1.77
0.05	1.11
0.06	1.05
0.07	1.34
0.08	1.43
0.09	1.34

Mg. Antigen	Vol. % x 10
0.075	1.66
0.100	2.64
0.125	3.20
0.150	4.00
0.175	5.74
0.200	6.98
0.225	9.39
0.250	10.0
0.275	7.42
0.300	7.23
0.325	8.77
0.350	12.14

irrespective of which reactant was varied and which was held constant. If varying, ascending amounts of antibody were added to a unit of antigen the curve had a slightly different shape than if the antibody were held constant and the antigen added in ascending amounts.

Many attempts were made to alter the course of the curve by altering the physical conditions surrounding the reaction. The amount of electrolyte in which the reaction occurred was varied, the pH was varied, and the tubes were shaken continuously throughout incubation, but all with a similar result. The overall height of the curve was changed with these changes of conditions but the shape remained the same, and the break and wander came at approximately the same ratio of antibody to antigen.

Ultimately the hematocrit tubes were discarded and a method of comparison was devised which, though it did not result in a curve, gave an accurate end point at the equivalence zone. This method was found to be useful not only for comparing the antigenic activity of fractions of Bact. tularense, but also for comparing the antibody content of various lots of tularense immune serum. The latter comparisons were made in conjunction with serum protection tests in rats, and the results of the antibody titrations *in vitro* were in good agreement with the respective survival rates of challenged rats. (Foshay, Ruchman and Nicholes (35).)

The tests were set up in a series of tubes so that antibody was mixed with antigen in varying proportions. If two or more antigens were to be compared a constant amount of each was set up against varying quantities of a given antiserum. If two or more antisera were to be compared a constant amount of each was set up against varying quantities of a given antigen. In each case the volume in all the tubes was kept constant by addition of saline solution. Table XVI and table XVII illustrate the usual additions of reagents in the reaction tubes. The tubes were well mixed by shaking and were incubated for 2 to 3 hours at 37°C. They were then centrifuged to throw down the precipitates and the supernatants were divided and tested for the presence of both excess antibody and antigen. The concentration of the varying reactant at which neither was present in excess or at which both were present in slight excess was considered to be the end point and the equiva-

Table XVI

No.	Mg. Antigen	Ml. Antigen Solution	Antibody Solution	NaCl Solution	Total Ml.
1	0.01	0.1	0.5	1.9	2.5
2	0.02	0.2	"	1.8	"
3	0.03	0.3	"	1.7	"
4	0.04	0.4	"	1.6	"
5	0.05	0.5	"	1.5	"
6	0.06	0.6	"	1.4	"
7	0.07	0.7	"	1.3	"
8	0.08	0.8	"	1.2	"
9	0.09	0.9	"	1.1	"
10	0.10	1.0	"	1.0	"
11	0.11	1.1	"	0.9	"
12	0.12	1.2	"	0.8	"
13	0.13	1.3	"	0.7	"
14	0.14	1.4	"	0.6	"
15	0.15	1.5	"	0.5	"
16	0.16	1.6	"	0.4	"
17	0.17	1.7	"	0.3	"
18	0.18	1.8	"	0.2	"
19	0.19	1.9	"	0.1	"
20	0.20	2.0	"	0.0	"

Antigen Solution

0.1 mg./ml. of solution

Antibody Solution

0.2 ml. serum/ml. of solution

NaCl

0.9% solution

Addition of Reagents to Determine Antibody Index

Table XVII

Series A	Series B	Ml. Antiserum Solution	Ml. Antigen Solution	Ml. Saline Solution	Total Ml.
01	1	0.1	0.5	0.9	1.5
02	2	0.2	"	0.8	"
03	3	0.3	"	0.7	"
04	4	0.4	"	0.6	"
05	5	0.5	"	0.5	"
06	6	0.6	"	0.4	"
07	7	0.7	"	0.3	"
08	8	0.8	"	0.2	"
09	9	0.9	"	0.1	"
1	10	1.0	"	0.0	"

Series A

For antiserum additions use as indicated a 1:100 dilution of the standard antiserum.

Series B

For antiserum additions use as indicated a 1:10 dilution of the standard antiserum.

The Antigen Solution

Contains 0.1 mg./ml. of solution of the antigen to be tested.

The A Series is to be used where the activity of the antigen per ml. falls below the range measured by Series B.

Addition of Reagents to Determine the Antigen Index lence point of this antigen-antibody system. In most cases the end point was very sharp. Table XVIII records the results of several tests of the phenol and water soluble antigen against various antisera. Table XIX records several tests of one antiserum against some of the different active antigens obtained by the described fractionation methods. All fractions are not represented since some had been entirely depleted by other experiments but all fractions which showed activity, and were still available, were tested.

Each antigen and each antiserum was given an index number which represented a ratio of the reactants at the equivalence point. This number was derived as follows:

$$Ai = \frac{G}{A} \qquad (1)$$

where G is the amount of antigen in milligrams necessary to precipitate A milliliters of the antiserum at the equivalence point, and Ai is the index number for that serum. Also,

$$Gi = \frac{G}{A} \qquad (2)$$

where A is the amount of antiserum in milliliters sufficient to precipitate G milligrams of the antigen at the equivalence point, and Gi is the index number for that antigen.

Table XVIII

Mgs. Antigen	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13	0.14	0.15	0.16	0.17	0.18	0.19	0.20	
G/S										+	+	-	-	-	-	-	-	-	-		L-44-G
S/A										-	+	+	+	+	+	+	+	+	+		0.1 ml
S/A	-	-	-	-	+	+	+	+	+	+	+	+									#50737-H
G/S	+	+	+	+	+	-	-	-	-	-	-	-									0.1 ml
S/A	-				-	-	-	±	+	+	+	+	+	+							L-45-A
G/S					+	+	±	-	-	-	-	-	-	-							0.1 ml
S/A					-	-	-	-	-	-	±	+	+	+							L-44-E
G/S					+	+	+	+	+	+	±	-	-	-							0.1 ml

Mgs. Antigen	0.001	0.002	0.003	0.004	0.005	0.006	0.007	0.008	0.009	0.010	
S/A	-	-	-	-	-	-	-	±	+	+	#79409-G
G/S	+	+	+	+	+	+	+	+	-	-	0.1 ml.
S/A	-	±	+	+	+	+	+				Normal Horse
G/S	-	-	-	-	-	-	-				0.1 ml.

S = Supernatant; A = Antiserum; G = Antigen
\+ = Positive ring test; - = Negative ring test

Equivalence Point of Various Immune Sera against $Ph-H_2OS$

Table XIX

Ml. Antiserum	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.10	
S/A	+	+	+	+	-	-	-	-	-	-	$Ph-H_2OS$
G/S	-	-	-	±	+	+	+	+	+	+	0.05 mg.
S/A	+	+	±	-	-	-	-	-	-	-	Di-3FDAI
G/S	-	-	±	+	+	+	+	+	+	+	0.05 mg.
S/A	+	+	±	-	-	-	-	-	-	-	$Ph-H_2OS$ (Alc. ppt.)
G/S	-	-	±	+	+	+	+	+	+	+	0.05 mg.
S/A	-	-	-	-	-	-	-	-	-	-	$Ph-H_2OSH$
G/S	-	+	+	+	+	+	+	+	+	+	0.05 mg.

Ml. Antiserum	0.001	0.002	0.003	0.004	0.005	0.006	0.007	0.008	0.009	0.010	
S/A	+	+	+	+	+	-	-	-	-	-	*$Ph-H_2OS$ (Alc. ppt.)
G/S	-	-	-	-	±	+	+	+	+	+	0.05 mg.

* Antigen was allowed to stand in the alcohol 7 days during precipitation.

S = Supernatant; A = Antiserum; G = Antigen
+ = Positive ring test; - = Negative ring test

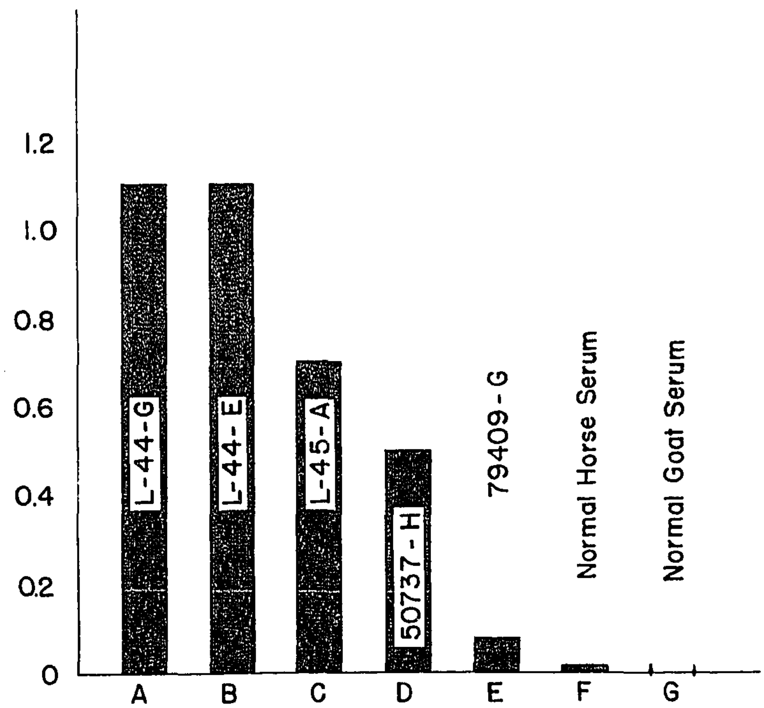
Equivalence Point of Various Antigen Fractions against Tularense Immune Serum L-44-E

Table XX

Mgs. Antigen	.01	.02	.03	.04	.05	.06	.07	.08	.09	.10	.11	.12	.13	.14	.15	.16	.17	.18	.19	.20	.25	.30	.35	.40	.45	
S/A	-	-	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	L-44-G
G/S	+	+	∓	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	-	-	-	0.1 ml.

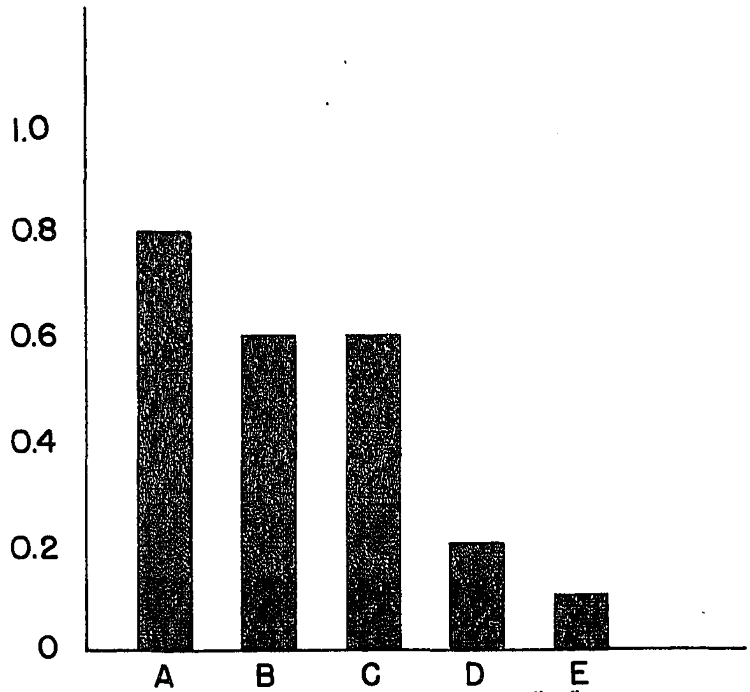
S = Supernatant; A = Antiserum; G = Antigen
+ = Positive ring test; - = Negative ring test

Equivalence Point of Fraction Ph-H_2OS-AS against Tularense Immune Serum L-44-E



A Linear Comparison of "Ai"
of Various Antisera

Figure 8



A Linear Comparison of "Gi" of Various Fractions

Figure 9

A - Ph-H_2OS

B - Di-3FDAI

C - Ph-H_2OS (Alcohol precipitated)

D - Ph-H_2OSH

E - Ph-H_2OS (Alcohol precipitated. Fraction was allowed to stand in alcohol for sometime.)

Figures 8 and 9 are linear comparisons of the tested antisera and antigens respectively.

In only one instance did the test fail to give a sharp equivalence point. The phenol, water and alcohol soluble fraction showed an excess of both antigen and antibody throughout the range from 0.02 mg. to 0.30 mg. of antigen against 0.1 ml. of antiserum. (Table XX)

It was noted in the introduction that complement fixation by any antigen-antibody system of Bact. tularense had not been adequately explored. Downs (1-b) observed that there was no wholly suitable antigen for the test. The whole bacterial cell manifests an anticomplementary effect and, therefore, reduces the sensitivity of the reaction. The antibody for the system presents the same problem. Many antisera from various sources were observed by Downs to be anticomplementary, with human serum showing this property the least.

It seemed possible that the use of the Ph-H_2OS fraction as an antigen might eliminate the anticomplementary effect observed with bacterial cells. Complement fixation tests were performed with it as antigen. Since the union of antigen and antibody formed a precipitate it was necessary to centrifuge the tubes so that readings could be made without the confusing turbidity caused by the precipitate. The degree of hemolysis was judged (a) by the intensity of the color of the supernatant fluids, and (b) by comparison of the sedimented erythrocytes in the centrifugates.

Table XXI illustrates a typical complement fixation test in which increasing increments of antigen were added to a constant amount of antiserum (0.14 ml.) and made to a constant volume of 1.0 ml. with saline solution. Antiserum in higher concentration per unit volume was anticomplementary. The antigen was not anticomplementary at any concentration. Fixation was not complete, but almost so. An increase in quantity of antigen did not increase fixation; on the contrary, as the concentration of the antigen increased above 0.1 mg. less complement was fixed. It was indicated that the optimal amount of antigen was between 0.09 and 0.10 mg. of antigen for 0.14 ml. of antiserum (L-44-E). If only 1 to 1.5 units of complement had been added it is possible that complete fixation would have occurred.

Table XXI

No.	Ml. Antigen	Ml. Antiserum	Saline	Complement	Sensitized Cells	Results
1	0.02	0.14	0.34	0.30	0.2	0.5 +
2	0.04	0.14	0.32	0.30	0.2	0.5 +
3	0.06	0.14	0.30	0.30	0.2	1.0 +
4	0.08	0.14	0.28	0.30	0.2	1.0 +
5	0.10	0.14	0.26	0.30	0.2	2.0 +
6	0.12	0.14	0.24	0.30	0.2	2.0 +
7	0.14	0.14	0.22	0.30	0.2	2.5 +
8	0.16	0.14	0.20	0.30	0.2	3.0 +
9	0.18	0.14	0.18	0.30	0.2	3.5 +
10	0.20	0.14	0.16	0.30	0.2	3.5 +
11	0.20	-	0.30	0.30	0.2	0 +
12	-	0.14	0.36	0.30	0.2	0 +
13	-	-	0.50	0.30	0.2	0 +
14	-	-	0.80	-	0.2	-

Complete fixation is indicated by the number 4+.

Antigen Solution
0.5 mg./ml.

Antiserum Solution
Undiluted

Complement
1 unit/0.15 ml.

Complement Fixation Test

DISCUSSION

Methods have been devised and described whereby certain substances which manifested antigenic activity were fractionated from Bact. tularense. Methods were also devised and described for comparative measurement of the antigenic activity of these substances, and some practical uses for the fractions have been demonstrated or indicated.

It was necessary to attack the bacterial cell in order to obtain active substances. The slight activity which was demonstrated in a dialyzed culture filtrate might be explained by experiments of Foshay, Downs, Ruchman and Nicholes (29) who found that a fine sintered glass filter did not remove all the organisms from a culture supernatant. These organisms were viable and therefore can hardly be called a fraction of Bact. tularense. It was probably an aggregation of these tiny reproductive units at the interface of the immune serum and culture supernatant that caused the positive reaction. No precipitin reaction was observed with non-dialyzed culture filtrates. The reason for this, and for the inability of immune serum to agglutinate Bact. tularense in the presence of this medium, is not known. The salt concentration of the medium was high (approximately 0.26 Molar NaCl) but, judging from the experience of many workers with different antigen-antibody systems, it was not so high that it would block agglutination completely. It seems more probable that other constituents of the medium are responsible for the failure to agglutinate.

Although mild treatment of Bact. tularense with acetone did not appreciably alter antigenicity it did alter its agglutinability by immune serum. Continued rigorous treatment with acetone altered its antigenicity, and its agglutinability was diminished to an even greater degree. This phenomenon (loss of agglutinability of the cells after treatment with various solvents and oxidation procedures) was observed by Foshay and Eigelsbach (30). They immunized rabbits to several differently prepared antigens, and found that even though the antisera from these animals would agglutinate formalinized cells to a high titer, they apparently would agglutinate their homologous antigens to only a very low titer or not at all. Despite this they showed that these treated cells were able to absorb antibody quantitatively from immune sera.

Cohn (31) demonstrated the same phenomenon after treating Proteus OX_{19} with benzene sulfonyl chloride. Immune serum which would normally agglutinate native organisms would not agglutinate the treated organisms. He further determined that an antigen-antibody complex was formed even though agglutination failed to occur, and he concluded that treatment with benzene sulfonyl chloride altered the surface to such an extent that "lattice" formation did not take place and thus aggregation did not occur.

In all probability the effect of treatment of Bact. tularense with acetone, nitrous acid, or phenol affects some surface feature of the bacterial cell which is closely related to agglutination, but that this alteration is not especially related to the union of antigen and antibody. It is of interest to point out that a formalin treated suspension of Bact. tularense is the standard agglutination antigen used in the diagnostic laboratory agglutination procedure, and since formalin has an affinity for amino groups that in all probability the amino group does not play an important role in the antigen-antibody complex of *Bact. tularense*. Further studies of the agglutination reaction in the presence or absence of formalin, and in the presence of various concentrations of formalin may indicate whether or not this deduction is correct.

The cells of *Bact. tularense* were successfully attacked by digestion with trypsin. Physical and chemical manipulation of the products of digestion yielded both immunologically active and inactive products. Only one of the active products showed immunogenic properties; the fraction called Di-3F. After dialysis this same substance failed to stimulate antibody production in rabbits. At no time thereafter, nor with any subsequent fraction, was antibody production demonstrated. The question arises, Why was the fraction Di-3F antigenic *in vivo*, whereas Di-3FD was not? The dialyzate from Di-3F manifested no activity *in vitro* and, therefore, no attempt was made to establish a reaction *in vivo*. Failure to do this is now perceived to have been an error because now it is not known if there was an immunogenic dialyzable substance present in Di-3F. Spies, Bernton, and Stevens (32) isolated a dialyzable immunogenic substance from cottonseed. Before dialysis it was a protein-polysaccharide complex. After dialysis an inactive polysaccharide remained in the collodion sac, and an immunogenic substance which gave positive tests for protein was found in the dialyzate. Coulson, Spies, and Stevens (33) later compared the dialyzability of their immunogenic, proteic product with the dialyzability of ovalbumin, and arrived at the conclusion that the immunogenic substance was freely diffusible through a collodion membrane. It is possible that a diffusable, antigenic molecule was present in the fraction Di-3F. It seems doubtful if this substance could have been immunogenic since most efficiently immunogenic substances are of large molecular size.

It is generally agreed that a large molecule is a better antigen than a small one, and that among antigenic substances the larger the molecule the better the antigen. Particle sizes may be increased by adsorption of a weakly antigenic molecule upon a larger molecule or particle such as collodion or charcoal, thus increasing the antigenicity of a poor antigen. Boyd and Malkiel (34) adsorbed crystalline horse hemoglobin onto killed bacteria, and produced therewith a specific antibody for the hemoglobin. Boyd (28) stated, "Even merely suspending particulate material, especially killed microorganisms in a solution of an antigen may suffice to enhance the antigenic stimulus so that fair antisera may be obtained against poor antigens such as hemoglobin." An analogous situation may have existed with respect to the digest fraction used to immunize rabbits. It was noted that Gram negative organisms were present as contaminants in the injected suspensions. It is possible that either the active carbohydrate fraction, or a dialyzable protein fraction, was adsorbed on the bacteria and that this caused the specific antigenic stimulus.

The digestion itself was quite successful in breaking down the organisms since 79.76% of the dried organisms was digested to a molecular size which would pass a cellophane membrane. The dialyzate contained, therefore, by far the greatest part of the organisms in the form of salts and substances which were positive to the usual tests for proteins.

Not much was learned of the fraction Di-3FDAS$_1$. It was negative to tests for protein and carbohydrate, yet it contained a slight activity. This could have been by contamination from the active fraction. A small amount which had remained soluble from the first precipitation with ethanol could have been present in too small a quantity to give a positive chemical test for carbohydrate. Immunologic reactions have long been known to be more sensitive than many chemical tests.

The two fractions, Di-3FDAI and Di-3FDAS$_2$, in light of the experience with the ethanol solubility of fraction Ph-H_2OS in the presence of sodium acetate and sodium hydroxide, were undoubtedly the same material. The immunologic activity, the chemical characteristics so far as they became known, and the physical characteristics of the 2 fractions were alike except for the respective solubilities in ethanol. It is probable that the digest fraction would also have proved to be insoluble in ethanol if a salt such as sodium acetate had been present at the time precipitation was attempted.

A second successful attack on the bacterial cell was made with concentrated phenol. Palmer and Gerlough (22) found that the capsular polysaccharides of most types of pneumococci were insoluble in concentrated phenol. Polysaccharides which were also soluble in phenol were recovered from phenol solutions by fractional precipitation with alcohol or glacial acetic acid. The immunogenic substances were isolated from the phenol insoluble polysaccharide. They also obtained an immunogenic phenol insoluble polysaccharide from typhoid bacilli after treating acetone extracted organisms with concentrated phenol. Both of these immunogenic polysaccharides were found in the phenol insoluble fractions.

Phenol extraction of Bact. tularense, however, did not yield an immunogenic phenol insoluble carbohydrate. On the contrary it yielded an immunologically inert substance which reacted positively to tests for carbohydrates and negatively toward tests for proteins and was, therefore, judged to be a carbohydrate, probably a polysaccharide.

The phenol soluble-water insoluble fraction, PhS-H_2OI, was judged to be a protein constituent of the organisms since all tests for protein were positive. One would have reason to expect that this fraction might have been antigenic. However, it was not. It is known that chemically induced changes in proteins may alter specificity and even destroy their antigenicity. It is probable that the treatment to which this protein was subjected destroyed its immunogenic character. Less drastic measures of extraction would probably have yielded an immunogenic protein from Bact. tularense. A complete study of the antigenic structure of the organism would necessitate such a trial. Extraction with diethylene glycol, as carried out by Morgan (19) with B. dysenteriae Shiga, might yield a protein which was not denatured to the point of complete loss of antigenic reaction.

Immunologic activity was found in a phenol soluble-water soluble fraction, Ph-H_2OS, and in what was designated as a phenol soluble-water soluble heavy fraction, Ph-H_2OSH. Except for particle size, and a different intensity of immunologic activity of each, both fractions were alike. It is believed that the 2 fractions are identical, the only difference being one of size of particle. If this assumption is correct it must be assumed that an intermolecular attraction existed which tended to aggregate the molecules into particles of varying size, some becoming large enough to be separable by intensive centrifugation. This aggregation to a larger particle size would probably lower the number of immunologically active groups which would otherwise have been available, and would undoubtedly diminish greatly the surface area in relation to mass. The only available active patches would be those turned to the surface of the particle. If the above were correct then one would expect a lower antigen index per unit of mass of the larger than of the smaller particle. The antigen index investigations proved that this was true.

It might be postulated that the particles vary in size owing to variation in the extent of polymerization of the carbohydrate. One might also postulate a glucosidic linkage through an aldose group of at least one of the possible different types of carbohydrate molecules which make up the complex, and that an aldose group is left free in each polymerized grouping. This is based upon the known facts that the unhydrolyzed complex is a reducing substance, and that after hydrolysis with strong HCl and heat more reducing substance is released.

The nitrogen present in the complex appears to be closely associated with antigenic activity since the progressive reduction in nitrogen content observed after serial precipitation with ethanol resulted in progressive reduction of immunologic activity.

Accurate methods for the quantitation of small differences between various antigens and between different immune sera were needed inasmuch as no such methods were available previously. The antigen and antibody index determination methods were devised as means of appraising the activity of an antigen, and of measuring the antibody content of an immune serum.

The failure to measure accurately and directly the volumes of specific precipitates by means of capillary tubes was attributed to the physical state of the precipitate formed at different proportions of antigen to antibody. In the zone of great excess of either antigen or antibody the "lattice-like" particles of the precipitate were small and fairly constant in size so that irregularity of the interstitial spaces of the packed precipitates did not contribute to inaccuracies in measurements of their volumes. But as the proportion of the reactants approached the equivalence zone the lattices formed into ever larger and more irregularly shaped, rigid floccules. This prevented the formation of regular interstices of the packed precipitate and it is believed that this variable volume of specific precipitates was the feature that made the method worthless.

The determination of the equivalence point by demonstrating that there existed neither an excess of antigen nor of antibody appears to be an accurate method for quantitating either antigen or antibody.

The quantitation of antibody was accomplished best by varying the antigen against a constant amount of antibody. The quantitation of antigen was achieved best by varying the antibody against a constant amount of antigen.

The one instance in which a sharp end point was not obtained was puzzling but it is possible to offer an explanation for the phenomenon. Reactions between antigen and antibody may be reversible at equilibrium. It is suggested that the reaction at the equivalence point is

$$A + G \rightleftarrows AG$$

and that here 3 compounds, A, G, and AG, are present. If the data shown in tables XVIII and XIX are examined closely, it will be noted that in most determinations there was a slight overlapping of the excess of both antigen and antibody, so that a plus sign appears in both columns simultaneously at the equivalence point. In all cases except the one under discussion this zone was very narrow. It seems possible that in the antigen-antibody system in which the Ph-H_2OSAS fraction was the antigen the product AG dissociated in the region of great antibody excess and also in the region of great antigen excess.

An alternative explanation is offered by the assumption that 2 antigen-antibody systems were involved, one with a high antibody index and another with a low antibody index. The summation of the resultant effects of these 2 systems could account for the picture illustrated in table XXII.

It is recognized that this study of the antigenic structure of Bact. tularense is incomplete, and that the work herein described is preliminary or exploratory but that it provides a basis for further work toward a more complete knowledge of the immunology of Bact. tularense. Although it is an introductory study of the antigenic structure of the organism it has provided initial methods and tools for future

Table XXII

Mgs. Antigen	System A		System B		Summation	
	S/A	G/S	S/A	G/S	S/A	G/S
0.01	−	+	−	+	−	+
0.02	−	+	−	+	−	+
0.03	−	+	±	−	±	+
0.04	−	+	+	−	+	+
0.05	−	+	+	−	+	+
0.06	−	+	+	−	+	+
0.07	−	+	+	−	+	+
0.08	−	+	+	−	+	+
0.09	−	+	+	−	+	+
0.10	−	+	+	−	+	+
0.11	−	+	+	−	+	+
0.12	−	+	+	−	+	+
0.13	−	+	+	−	+	+
0.14	−	+	+	−	+	+
0.15	−	+	+	−	+	+
0.16	−	+	+	−	+	+
0.17	−	+	+	−	+	+
0.18	−	+	+	−	+	+
0.19	−	+	+	−	+	+
0.20	−	+	+	−	+	+
0.25	−	+	+	−	+	+
0.30	+	±	+	−	+	±
0.35	+	−	+	−	+	−
0.40	+	−	+	−	+	−
0.45	+	−	+	−	+	−

An illustration of the possibility of two antigen-antibody systems present in a given antigen and a given antiserum. When System A and System B are together the resultant picture would be as illustrated in the column at the right.

work, possibly also useful for studies on other antigen-antibody systems.

SUMMARY

A technique was devised for mass cultivation of Bact. tularense. Improvements were made in methods for preparation of certain constituents of the liquid medium which resulted in increased yields of Bact. tularense.

Chemical fractions of Bact. tularense, both immunologically active and inert, were obtained by 2 methods, tryptic digestion of acetone extracted organisms with subsequent physical and chemical manipulations which yielded an inert dialyzable fraction and an active alcohol insoluble fraction; and the extraction of acetone treated organisms with phenol with subsequent physical and chemical manipulations which yielded an inert phenol and water insoluble carbohydrate, an inert phenol soluble and water insoluble protein, an active phenol soluble-water soluble carbohydrate, and an active phenol soluble-water and alcohol soluble substance which could not be classified.

An in vitro method was devised to measure quantitatively the activity of antigenic sub-

stances. This method can be applied to give index figures for the activity of an unknown antigen against a given antiserum, or for an unknown antiserum against a standardizable antigen.

The experiments with complement fixation were only introductory but they indicate another practical use for antigenic fractions made available by the extraction methods described above.

One isolated fraction proved to be a more suitable antigen for complement fixation than any material heretofore available.

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