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ANTIGENIC RELATIONSHIPS OF SOME ORGANISMS OF THE GENUS  
PASTEURELLA AS DETERMINED BY THE ANTIGEN-ANTIBODY  
DIFFUSION TEST

by

LLOYD LAVERNE DAHL

B.A., Carroll College, Helena, Montana, 1950

Presented in partial fulfillment  
of the requirements for the degree of  
Master of Science

MONTANA STATE UNIVERSITY

1952

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L. L. D.

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## INTRODUCTION

In thermoprecipitin reactions of Pasteurella pestis in tissues of animals dead with plague, cross reactions among P. pseudotuberculosis, P. multocoda and B. tularensis indicate that all the organisms have antigens in common (29). From the standpoint of serology it would be interesting to know how many are present and if any are unique. This knowledge would be of value not only in serological diagnosis, but also in the classification of the organisms in question.

The main objectives in this study are twofold: first, to determine whether an antigenic relationship exists among Pasteurella tularensis, P. pestis and P. pseudotuberculosis and second to determine the number of antigens present in specified preparations of each.

A number of different immunological technics can be utilized to determine whether a material contains one or several antigens (9), but these methods give only a crude estimation of the number of individual components present. In recent years a simple technic for immunochemical analysis has been described by Oudin (24,27).

The method of Oudin consists of overlaying a solid immune serum-agar mixture in a thin bore tube with the antigen solution. The antigen-antibody precipitate is formed as a sharp band and moves down the tube as more antigen diffuses

into the agar. Oudin showed that only one band is formed when a single antigen-antibody system is present.

### Historical Resumé

Prior to Oudin's observations, Bechhold (1) in 1905 described a reaction between goat serum and antigoat rabbit serum in one-percent gelatin. Fornet and Muller (5) in 1910 had observed multiple bands using the interfacial technic in liquid media. A similar observation was made by Nicolle, Cesari and Debains (16) in 1920 for diphtheria toxin and horse antitoxin. Diphtheria toxin mixed with an equal part of ten-percent gelatin was placed in a tube and allowed to react with immune serum layered on the surface. In 1932 Petrie (28), Sia and Chung (30) and Kirkbride and Cohen (12) described both specific and non-specific ring phenomena around bacterial colonies growing on substrates containing immune serum. Their investigation concerned meningococci, pneumococci and Shiga dysentery bacilli. The method of Kirkbride and coworkers was later employed for the serological typing of meningococci and pneumococci. Ouchterlong (23) in 1948 described a diphtheria toxin-antitoxin reaction of a similar character.

Oudin's serum-agar technic has already been applied to demonstrate the antigenic complexity of horse serum (25), bovine cytochrome c (2) and ragweed pollen extracts (3). It has also been employed by Kabat and coworkers (8, 10) to

determine the serological homogeneity of antibodies to human globulin and albumin. For the purpose of defining antigen-antibody systems, antisera of crystalline protein were obtained, quantitative precipitin tests were performed, and the supernatants, after the removal of the precipitates, were tested for the presence of antigen and antibody to determine the homogeneity of the systems used (11). By applying the serum-agar technic to these same systems it was possible to compare the results obtained with the two methods. Oudin found that the number of bands correspond to the number of antigen-antibody systems of known compositions. Observations showed that the number of bands formed was determined by the number of known antigen-antibody systems present in the prepared mixtures, (15).

Ouchterlong (17, 22) has recently described and applied a technic using the same principle in which both antigen and antibody are allowed to diffuse in an agar medium.

The following diagrams (on the next page) might serve better to point out the reaction types in question. These were taken from the work of Bjorklund (4).

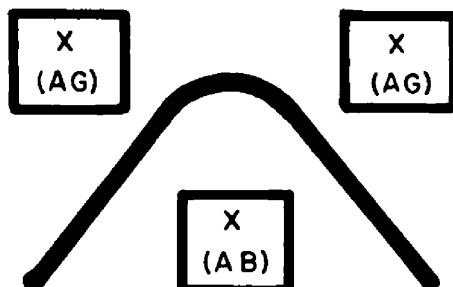


Figure 1

**Reaction of identity:** If two antigenic preparations, both containing the antigen X, diffuse from the top basins (AG) they will form a continuous line of precipitate with the anti-X-serum diffusing from the bottom basin (AB).

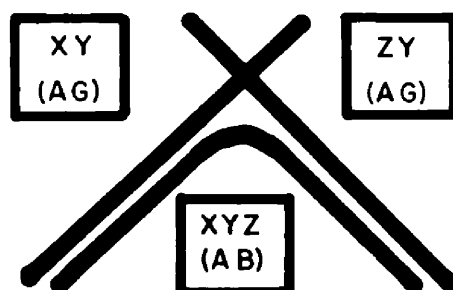


Figure 2

**Reaction of non-identity:** If two antigen preparations containing different antigenic components diffuse from the top basins each antigen will form a line of precipitate with the corresponding antibody from the bottom basin. The lines do not interfere with each other.

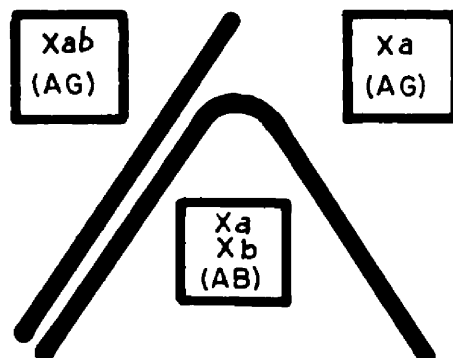


Figure 3

**Reaction of partial identity:** If one of the antigens has two antigenic properties, one of which is common with the other antigen, a partial fusion of the precipitates will occur.



## CHAPTER I

### MATERIALS

One strain of P. pestis (S22), one strain of P. pseudotuberculosis (M25), one strain of P. tularensis (B38), and one strain of E. coli B were employed in the study. The cultures and white rabbits came from stocks maintained at the Rocky Mountain Laboratory, Hamilton, Montana. Each bacterial strain gave typical reactions when tested on various sugars and other differential media.

The medium (pH 6.8) specified below was used to grow E. coli, P. pestis, and P. tularensis. For the growth of P. pseudotuberculosis the cystine was omitted.

Beef heart, infusion . . . . .	25 grams
Proteose peptone, Difco. . . . .	10 grams
Bacto-Dextrose . . . . .	10 grams
Sodium chloride. . . . .	5 grams
l-cystine. . . . .	1 gram
Bacto-agar . . . . .	15 grams
Distilled water. . . . .	1000 ml

A temperature of 30°C was maintained for cultivation of strains S22 and M25; B38 and E. coli were incubated at 37°C. Three transfers on slants of the above medium were made before planting the bacteria.

Cells were grown on  $\frac{1}{2}$  x 10 x 20 cm agar surfaces contained in flat glass bottles. An amount of cells equivalent

to one gram dry weight was harvested from the required number of bottles. The harvested material was suspended in three volumes of 0.85% NaCl and washed four times by centrifugation at 2000 G for thirty minutes. The resulting cells were suspended in a small amount of saline to give a thick creamy suspension. This suspension was treated for one hour with two volumes of acetone at room temperature. Dehydrated antigen was obtained by placing the acetone treated cell suspension on a Whatman forty filter paper over a Buchner funnel hooked on to a vacuum flask and allowing the cells to dry over night at room temperature. The dried cells were ground in a mortar, put into vials, and stored at - 20°C.

Disintegration of the antigen suspension was attained by mechanical means, employing a Mickle tissue disintegrator. A half gram of the dried cells were mixed with ten ml of a M/100 phosphate buffered 0.85% saline solution (pH 7.0); 5 ml of the antigen suspension was added to ten grams of glass beads plus two drops of tributyl citrate and allowed to vibrate for thirty minutes. During this procedure the temperature was recorded at 42°C. The resulting disintegrated mixture was pipetted off and centrifuged for one hour at 20,000 G to remove the sediment. The clear supernate was dialyzed four times at a temperature of 4°C against six hundred cc of M/100 phosphate buffered saline; changes of the dialyzing medium were made at forty-eight hour intervals. Nitrogen determinations were performed on each antigen after such dialysis.

Antigen concentrations were estimated by use of nitrogen values as determined by the micro-Kjeldahl method modified according to Ma and Zuazaga (12). The nitrogen values ranged from 0.29 to 0.64 mg N/ ml. All antigens were diluted to be equivalent to the antigen having the lowest nitrogen value, except in the case of P. tularensis in one part of the experiment.

The respective antisera were derived from rabbits inoculated intravenously with twelve 0.1 ml doses of the above described antigens; injections were made at two day intervals. Two days after the last injection the rabbits were anesthetized with Nembutal (sodium pentobarbital) and bled directly from the heart. The amount of blood removed from each rabbit was approximately forty ml. One week later the rabbits were bled again, and the respective sera pooled.

Precipitin titers of the various serums were determined by the capillary tube method (13). The titers were M25, 1:32; S22, 1:32; B38, 1:16 and E. coli B, 1:32.

To minimize contamination, the antisera and antigens were treated by ultraviolet radiation before the antigen-antibody diffusion tests were performed.

The diffusion medium employed for the tests was prepared as follows: 1.5% agar was added to a solution (pH 7.0) which contained M/100 phosphate buffer in 0.85% NaCl. This was heated and while hot it was centrifuged to remove the precipitates, leaving a very clear medium wherein diffusion reactions could be detected. The media was sterilized in the

autoclave and penicillin with streptomycin (200 units of penicillin and .5 mg of streptomycin) was added when the media had cooled to 45°C.

## CHAPTER II

### EXPERIMENTAL

#### General Introduction

Antigen-antibody reactions in agar are individual reactions between different antigens and antibodies. The principle of the antigen-antibody diffusion test is that a band of precipitate is formed between the antigen and antibody at some point where they meet, resulting in a line. These lines may be formed in different positions depending upon the nature of the antigen and antibody.

Diffusion can be utilized in two ways. One method is to incorporate the reagent (antibody or antigen) into the agar, and to allow the other reagent to diffuse producing a concentration gradient. The other method is to allow diffusion together of both reagents so that two concentration gradients are formed in the agar. The latter will be referred to as the double-diffusion gradient method. This has advantages over the first in that it produces conditions for the interaction of antigen and antibodies in all proportions in a complex system. Both antigen and antibody can be diffused into an agar base so that a concentration gradient develops by diffusion for an appreciable distance.

The complexity of antibodies in sera is a well known fact. It is also known that the antigen is often a complex made up of numerous chemical components. The double-diffusion gradient is comparable in its action to a prism, in that the constituent systems are separated into individual lines, as are the colors of the spectrum.

### Methods Employed

The experiments were performed in Petri dishes (approximately ten cm wide). A thin layer of the medium (approximately ten cc) was poured into each to obtain an even layer for diffusion. The agar was allowed to solidify and oxford cups were heated gently and placed on the surface of the agar one cm apart in the case of two cups on each plate. When five cups are on a plate the four outside cups are placed nearest the edges of the center cup, that is, one and seventh ten cm apart. (See figure 4). The center cup was filled with .5 ml of anti-serum and the four outer cups were each filled with five tenths ml of the designated antigen suspension. The cups were refilled whenever the reagents had diffused. This was done twice. The petri dishes were placed in a humidified incubator at 30°C until precipitation was visible. Visible precipitation bands were formed in eight to ten days. The visible lines were counted by viewing the plates under oblique transmitted light.

In the basin technic the basins were made by removing  $\frac{1}{2} \times \frac{1}{2}$  inch squares of the solidified medium. They were placed

five-eighth of an inch apart from the top corners of the bottom basin (see figure 7).

Duplicate plates with and without penicillin and streptomycin were tested to determine if it had any effect upon the number of lines produced. It was found that the number of lines were the same in both cases.

A number of different arrangements of the antigen (AG) and the antibody (AB), using the oxford cups and basins were tested. The basin method was found to be practical but the oxford cup technic appeared as the most convenient

E. coli was employed as a control organism supposedly unrelated to the members of the genus Pasteurella. E. coli was thus used as a test for non-specific antigenic materials which might be derived from the medium and be present in the antigens prepared as described. Antibody against E. coli was tested against antigen prepared from E. coli and from P. pseudotuberculosis as well as the basic broth medium containing either glucose or cystine. As figure 4 indicates there was no reaction with the basic medium, thus no demonstrable non-specific antigenic materials were derived from the medium. The five lines formed between E. coli AB and M25 AG indicates that there are antibodies present which react with antigens in M25 AG. These lines indicate the number of cross reactions. The reaction between the antigen of E. coli and its specific antiserum AB shows eleven lines, which correspond to eleven antigenic components.

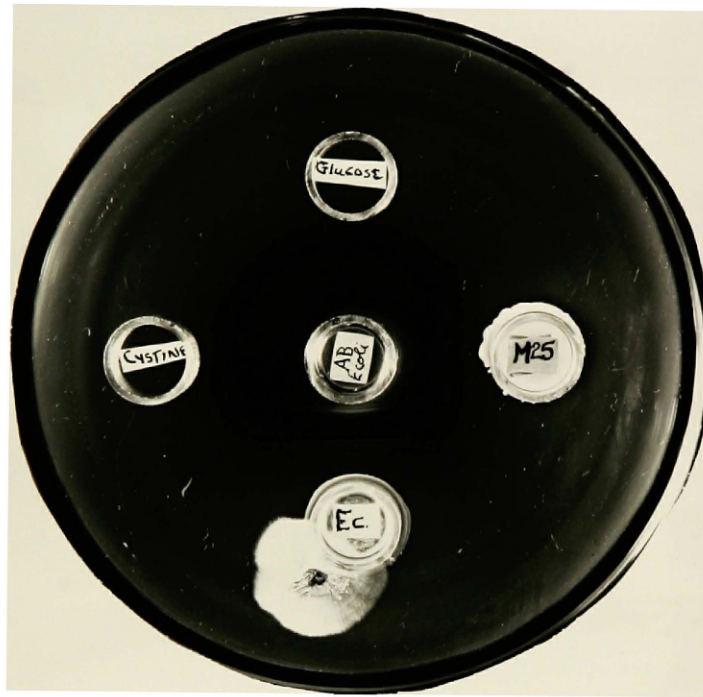


Figure 4. Test for non-specific materials derived from the medium.



## CHAPTER III

### RESULTS

1. Demonstration of the number of antigenic components with homologous systems.

#### Test 1. P. tularensis

On a surface of agar two oxford cups were placed at a distance of one cm from each other. One of the cups was filled with five tenths ml of B38 antiserum (AB) and the other with five tenths ml of B38 antigen. They were refilled twice. The plate was held at 30°C and after eight days band-like precipitation was visible. Numerous plates were studied and in all instances eight lines were noted. Figure 5 demonstrates the lines.



Figure 5. Demonstration of the number of anti-genic components detectable with homologous P. tularensis precipitin systems.

Test 2. P. pestis

The initial conditions were the same as in Test 1. The number of separate bands of precipitation was found to be at least ten. Figure 6 shows the appearance of this kind of reaction employing the oxford cups. Likewise in Figure 7 the same number of lines appeared using the basin technic of Bjorklund (4).

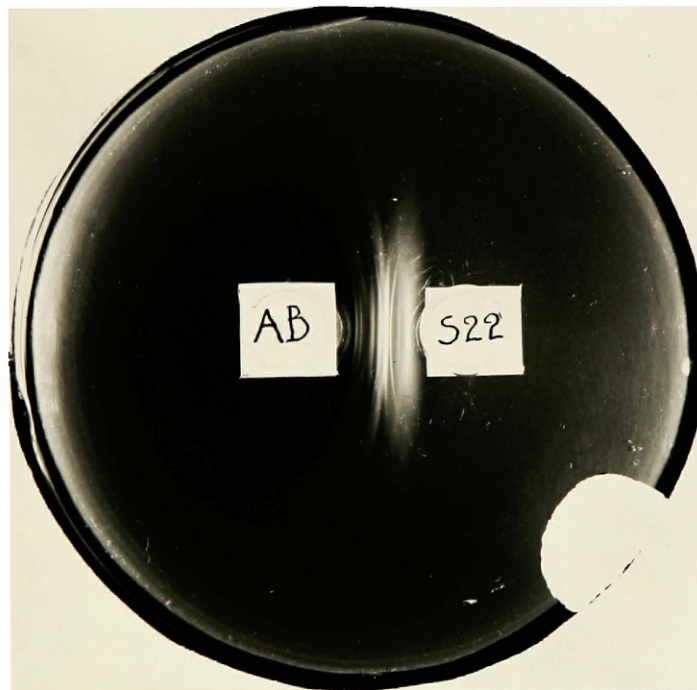


Figure 6. Demonstration of the number of antigenic components detectable with homologous P. pestis precipitin systems.

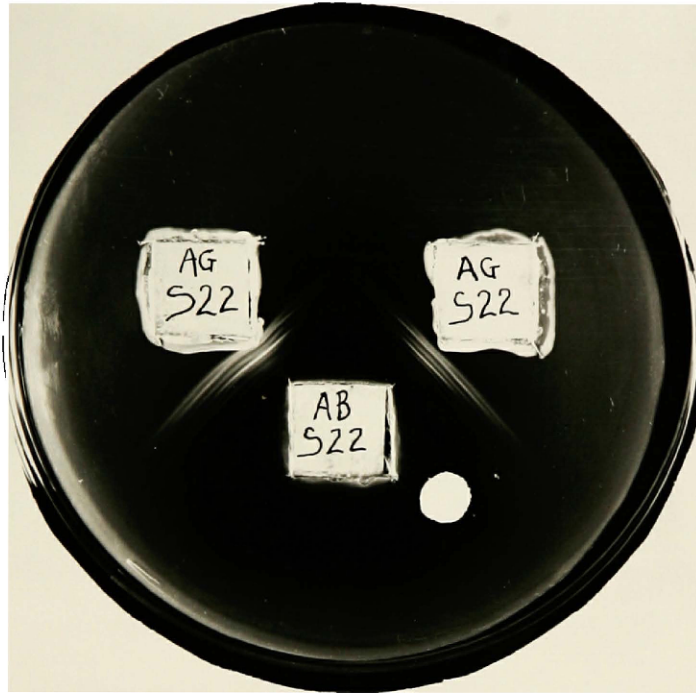


Figure 7. Demonstration of the number of antigenic components detectable with homologous P. pestis precipitin systems.

Test 3. P. pseudotuberculosis

Following the same set of conditions as in the prior tests the determinations of the number of lines present was found to be nine in the case of P. pseudo-tuberculosis. Figure 8 shows the appearance of this kind of a reaction.



Figure 8. Demonstration of the number of antigenic components detectable with homologous P. pseudotuberculosis precipitin systems.

#### Test 4. E. coli

Following the same set of conditions as in the other tests the determination of the number of lines present was found to be eleven in the case of E. coli. Figure 9 shows the appearance of this set of reactions.



Figure 9. Demonstration of the number of antigenic components detectable with homologous E. coli precipitin systems.

2. Demonstration of the number of antigenic components with heterologous systems.

#### Test 5. P. pestis

On a surface of agar five oxford cups were placed. The four outside cups are placed one and seventh tenths cm from the nearest edges of the center of the cup. The center cup was filled with five tenths ml of antiserum and the four outer cups with five tenths ml of the designated antigen. They were refilled twice. The plate was held in a humidified incubator at 30°C until precipitation was visible.



Figure 10 shows the cross reactions of various signi-  
fied antigens with the antibodies of P. pestis. The figure

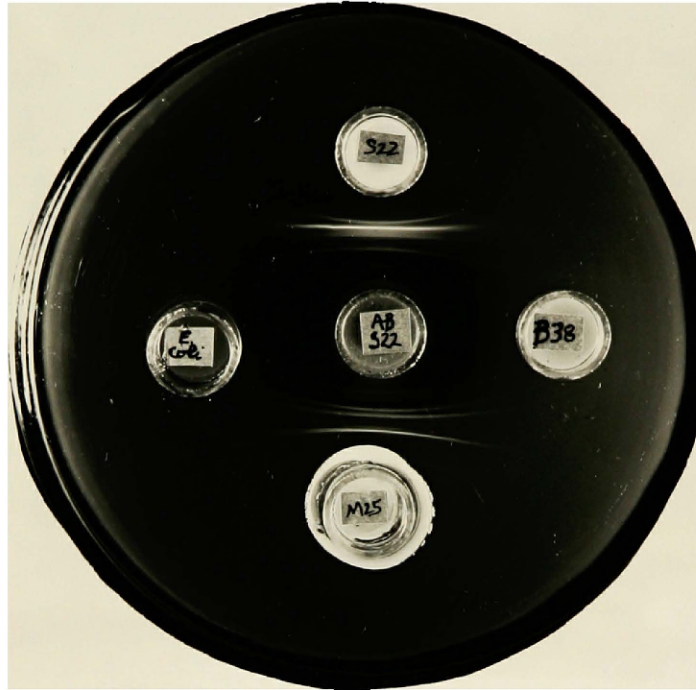


Figure 10. Demonstration of the number of antigenic components detectable with heterologous systems in the case of P. pestis antibody.

demonstrates a cross reaction between S22 (AB) and M25 (AG) involving at least eleven components. At least nine components are demonstrated with S22 (AG), at least one component with B38 (AG), and at least three components with E. coli (AG). The cross reaction that is evident does not necessarily mean that the antigens are common, but rather that antigens are present which are capable of reacting with antibodies formed in response to S22 antigen.

It is difficult to explain the greater number of components demonstrated with the heterologous M25 antigen. One possible explanation is that several lines are present in the rather broad bands of precipitate formed with S22 antigen. In this case, the number of distinct lines visible would be reduced.

Test 6. P. pseudotuberculosis

Figure 11 shows an heterologous system in respect to P. pseudotuberculosis antibodies. The figure demonstrates a cross reaction between M25 (AB) and M25 (AG) involving at least six components. At least five components are demonstrated with S22 (AG), at least three components with E. coli, and none are demonstrable with B38 (AG).

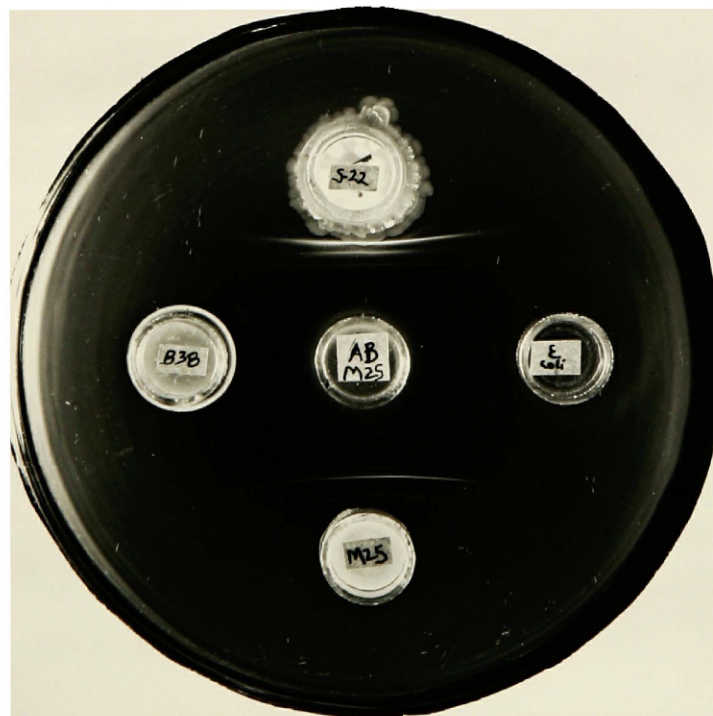


Figure 11. Demonstration of the number of antigenic components detectable with heterologous systems in the case of P. pseudotuberculosis antibody.



The number of components demonstrated in this particular test might be low in all cases because the homologous M25 antigen-antibody only show six components whereas nine are previously demonstrated (see Figure 8, page 17).

### 3. Demonstration of looping

The phenomenon of looping is of fundamental importance. When two or more samples of identical antigenic material are placed in proper position in the presence of homologous antibody the lines produced by these systems intercept and become continuous, that is, lines loop. Identical systems are considered to be those in which common antigenic material will produce intercepting lines in the presence of homologous antibody (see Figure 12).

This applies, however, only if the lines are formed at the same time. Lack of looping--and consequently erroneous interpretation--may occur if one of the lines is already well formed when a second approaches it.

#### Test 7. E. coli and P. tularensis

The initial conditions were the same as in the section dealing with heterologous systems (see test 5, page 18).

Figures 12 and 13 demonstrate looping in a homologous multiple system of known common antigens. It shows the appearance of the loops when the oxford cup technic is employed. It should be brought out that in the case of E. coli eleven

antigenic components were demonstrated in test 4, Figure 9, page 17, and that in Figure 12 these are not eleven complete loops. Likewise in Figure 13. This serves to illustrate looping may not be demonstrable with those components whose lines of precipitate are farthest removed from one another. Lack of looping, in these cases, is a problem of geometrical arrangement which was not explored under the conditions of these experiments.



Figure 12. Demonstration of looping in a known homologous system in the case of E. coli.

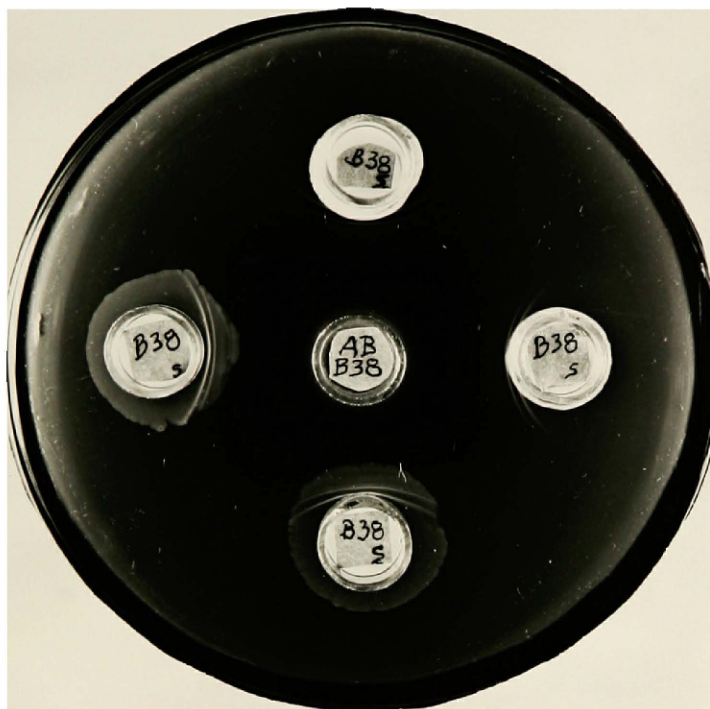


Figure 13. Demonstration of looping in a known homologous system in the case of P. tularensis.

Test 8. P. pestis and P. pseudotuberculosis

Figure 14 demonstrates the looping phenomenon of a reaction of partial identity in a heterologous system between P. pestis and P. pseudotuberculosis employing the basin technic of Bjorklund (4). There are ten lines formed between the antibody of P. pestis (S22) and its corresponding antigen (AG); while eight lines are formed with the antigenic components of P. pseudotuberculosis (M25). Of the eight formed two definitely loop. This is proof of at least two common antigenic components between the two organisms. The other six lines are considered to be cross reactions.

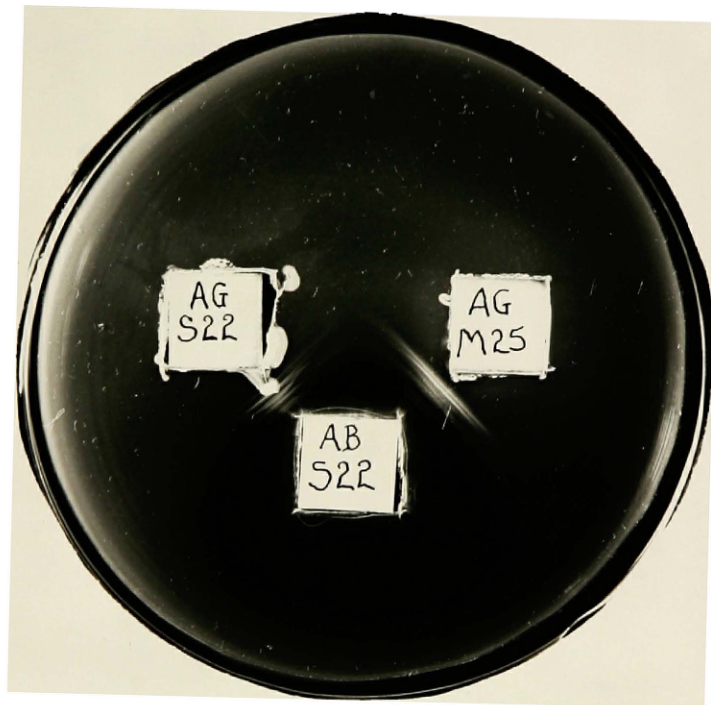


Figure 14. Demonstration of the two common antigenic components in P. pestis and P. pseudotuberculosis employing the basin technic.

Figure 15 also demonstrates that there are cross reactions between P. pestis (S22) and P. pseudotuberculosis (M25) and that two of the reactions definitely loop indicating common antigenic components.

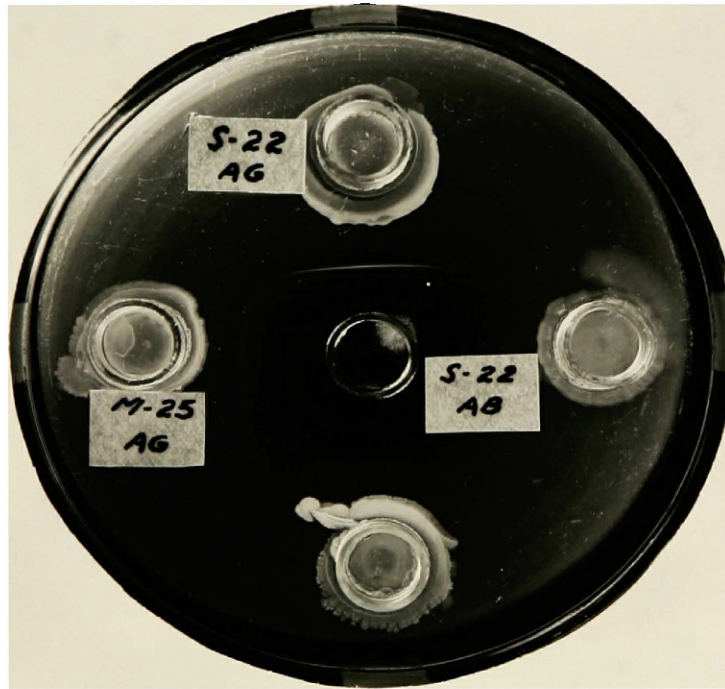


Figure 15. Demonstration of the two common antigenic components in P. pestis and P. pseudotuberculosis employing the oxford cup technic.

## CHAPTER IV

### DISCUSSION

Utilizing the antigen-antibody diffusion test it has been shown that there are at least eight antigenic components in a preparation of P. tularensis, at least ten antigenic components in P. pestis, at least nine antigenic components in P. pseudotuberculosis, and at least eleven antigenic components in E. coli. It should be noted that this might vary according to the procedures used for preparing the antigen and demonstrating precipitation bands.

By means of these tests several separate observations may be made. As mentioned, the number of antigenic components in a given system may be demonstrated. Also employing a given antiserum, the extent of antigenic cross reactions can be shown. These cross reactions may involve common components as demonstrated by looping or components which merely are capable of reacting with the antibodies formed in response to a given mixture of antigen.

Although only two common antigenic components were shown to exist in P. pestis and P. pseudotuberculosis, one must take into consideration the geometrical arrangements employed. Of the cross reactions which occurred in Figures 14 and 15 some of the reactions may have demonstrated looping if the cups had been differently arranged.

The two common antigens between P. pestis and P. pseudotuberculosis which were shown to exist are in agreement with the results of cross reactions which have long indicated strong similarity between P. pestis and P. pseudotuberculosis (29).

However, in the case of P. tularensis no such common antigens were shown to exist by looping. Recently some investigators have argued for the exclusion of this species from the genus Pasteurella (7), and assigning the species name of Bacterium tularense to it. The results of these tests would seem to confirm the validity of their contentions.



## CHAPTER V

### SUMMARY

1. A modification of Oudin's diffusion method for antigenic analysis is described.
2. The organisms and the number of antigens demonstrated in each were as follows: a) P. tularensis eight; b) P. pseudotuberculosis nine; c) P. pestis ten and d) E. coli eleven.
3. It was demonstrated that an antigenic relationship exists between P. pseudotuberculosis and P. pestis wherein two components are common.
4. P. tularensis has no common antigenic components in relation to P. pestis and P. pseudotuberculosis, but does have cross reactions.
5. The double-diffusion-gradient method presented here is essentially a qualitative approach effecting only a separation of the individual systems.



## BIBLIOGRAPHY

1. Bechhold, H. Zeitschrift fur physikalische chemie  
Vol. 52, 1905, Pp. 195.
2. Becker, E. and Munoz, J. Studies on the antigenicity  
of cytochrome c. Journal of Immunology. Vol. 63,  
1949, Pp. 173-181.
3. Becker, E. and Munoz, J. Multiplicity of antigens in  
ragweed pollen extracts as demonstrated by the technic  
of Oudin. Proceedings of the Society for Experimental  
Biology and Medicine. Vol. 72, 1949, Pp. 287-289.
4. Bjorklund, Bertil. Specific Inhibition of Precipitation  
As an Aid In Antigen Analysis With Gel Diffusion  
Method. Proceedings of the Society for Experimental  
Biology and Medicine. Vol. 79, 1952, Pp. 319-324.
5. Fornet, W., and Muller, M. Praktische und theoretische  
Präzipitinuntersuchungen. Ztschr. f. Hyg. u. Infect-  
ionskr. Vol. 66, 1910, Pp. 215-246.
6. Gray, George W. Electrophoresis. Scientific American.  
No. 185, Dec. 1951, Pp. 45-53.
7. Hesselbrock, W., and Foshay, Lee. The Morphology of Bact-  
erium tularense. Journal of Immunology. Vol. 49,  
1945, Pp. 209-236.
8. Kabat, E. A., Glusman, M. and Knaub, V. Quantitative Esti-  
mation of Albumin and Gamma Globulin in Normal and  
Pathologic Cerebrospinal Fluid By Immunochemical  
Methods. American Journal of Medicine. Vol. 4,  
1948, Pp. 653-662.
9. Kabat, E. and Mayer, M. Experimental Immunochemistry.  
Charles C. Thomas, Springfield. 1948.
10. Kabat, E. and Murray, J. P. A Comparison of Human Alpha  
Globulins in Their Reactivity With Rabbit and Anti-  
Alpha-Globulin By The Quantitative Precipitin Method.  
Journal of Biological Chemistry. Vol. 182, 1950,  
Pp. 251-259.
11. Kendall, F. E. Studies on Serum Proteins. I. Identi-  
fication of a Single Serum Globulin By Immunological

Means. Its Distribution In The Sera Of Normal Individuals and Of Patients With Cirrhosis Of the Liver and With Chronic Glomerulonephritis. Journal of Clinical Investigation. Vol. 16, 1937, Pp. 921-931.

12. Kirkbride, M., and Cohen, S. Precipitation Reactions Of Meningococcus Strains With Immune Serum In Agar Plates In Relation To Antigenic Activity. American Journal of Hygiene. Vol. 20, 1934, Pp. 444.
13. Larson, Carl L. Personnel communication, Rocky Mountain Laboratory, Hamilton, Montana, 1951.
14. Ma, T. S., and Zuazaga, G. A New Indicator and Improved Rapid Method Of Micro-Kjeldahl Determination Of Nitrogen. Annual Edition of Indian and English Chemistry. Vol. 14, 1942, Pp. 280.
15. Munoz, J. and Becker, E. E. Complexity of Antigen-Antibody Systems As Demonstrated By a Serum-Agar Technic. Journal of Immunology. Vol. 65, 1951, Pp. 47-57.
16. Nicolle, M. et. al. Annales De L'Institut Pasteur. Vol. 34, 1920, Pp. 596.
17. Ouchterlong, O. Antigen-Antibody Reactions In Gels. Arkiv for Kemi, Mineralogic och Geologi. Bd 26 B, No. 14, 1949, Pp. 1-9.
18.                     . Antigen-Antibody Reactions In Gels. II. Factors Determining The Site Of Precipitation. Arkiv for Kemi. Bd. 1, No. 9, 1949, Pp. 43-48.
19.                     . Antigen-Antibody Reactions In Gels. III. The Time Factor. Arkiv for Kemi. Bd. 1, No. 9, 1949, Pp. 55-59.
20.                     . Antigen-Antibody Reactions In Gels. Acta Pathologica Et Microbiologica Scandinavica. Vol. 26, 1949, Pp. 507-515.
21.                     . In Vitro Method For Testing Toxin Producing Capacity Of Diphtheria Bacteria. Acta Pathologica Et Microbiologica Scandinavica. Vol. 26, 1949, Pp. 516-524.
22.                     . An In-vitro Test Of The Toxin-Producing Capacity Of Corynebacterium diphtheria. Lancet. Vol. 1, n.d., Pp. 346-348.
23.                     . Acta Pathologica Et Microbiologica Vol. 25, 1948, Pp. 186.

24. Oudin, J. Immunology. Method Of Immunochemical Analysis By Specific Precipitation In Jellied Medium. Comptes rendus Academie des Sciences. Vol. 222, 1946, Pp. 115-116.
25. \_\_\_\_\_. Immunochemical Analysis Of Horse Serum By Means Of Specific Precipitation In Jellied Medium. Preliminary Results. Bulletin de la Societe de chimie biologique. Vol. 29, 1947, Pp. 140-149.
26. \_\_\_\_\_. Qualitative Immunochemical Analysis. Method By Diffusion Of Antigens In Gelose Precipitating Immune Serum. Part I. Annales De L'Institut Pasteur. Vol. 75, 1948, Pp. 30-51.
27. \_\_\_\_\_. Qualitative Immunochemical Analysis. Method By Diffusion Of Antigens In Gelose Precipitating Immune Serum. Part II. Annales De L'Institut Pasteur. Vol. 75, 1948, Pp. 109-127.
28. Petrie, G. F. A Specific Precipitin Reaction Associated With The Growth On Agar Plates Of Meningococcus, Pneumococcus, and B. Dysenteriae (Shiga). British Journal of Experimental Pathology. Vol. 13, 1932, Pp. 380.
29. Seal, S. C. Studies On The Specific Soluble Proteins Of Pasteurella pestis and Allied Organisms. Journal of Immunology. Vol. 67, August 1951, Pp. 93.
30. Sia, R. H. P. and Chung, S. F. Use of Antipneumococcus Serum Agar For The Identification Of Pneumococcal Types. Proceedings Of The Society For Experimental Biology and Medicine. Vol. 29, 1932, Pp. 792.