# STUDIED ON CLOSTRIDIUM BOTULINUM: IT'S OCCURRENCE IN THE SOILS OF KANSAS, AND THE EFFECT OF DIFFERENT TYPES OF SOIL ON TOXIN PRODUCTION.

By

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## INTRODUCTION.

Clostridium botulinum was recognized for the first time in 1895 by van Ermengen[1] who at that time was trying to discover the etiological factor underlying a severe epidemic of ham poisoning in Ellezelles, Germany. Van Ermengen not only isolated the organism from the ham, the ingestion of which had caused the outbreak, but he also recovered the same organism from the stomach and spleen of one of the persons who had died as a result of the poisoning. For many years following this discovery, the organism was not recognized in any country but Germany. In fact, it has only been within the last six or seven years that the Bacteriology of botulism has received much attention in the United States. Within that time, the increased number of home canned foods and the number of outbreaks of fatal poisoning occurring in this country, especially along the Pacific coast, have aroused much interest among investigators, with the result that the literature on this subject, both scientific and practical is growing rapidly.

In spite of previous investigations, it was not until the work of Gertrude S. Burke[2] in 1918, that the actual occurrance of Clostridium botulinum in nature was proved conclusively.

From a total of 235 cultures made, she found only seven which contained Clostridium botulinum and four in which

there was evidence of toxin but which was too weak to identify by the toxin-antitoxin test. These seven positive cultures were made from fruits, vegetables, spiders from bush bean plants, and moldy hay.

During the years 1920 to 1922, K. F. Meyer and B. J. Dubovsky demonstrated the presence of Clostridium botulinum, type B, in soil and vegetable specimens from Switzerland, the Netherlands, England, Belgium and Denmark[3], and in America, from the state of California[4]. From soil samples alone, they cultivated both types A and B from material secured in Alaska and Canada[4], while P. Schoenholz and K. F. Meyer[6] found both types in the soil of China and the Hawaiian Islands. In 1922, George E. Colemen[6] examined soils from a limited strip of Coast line in Santa Barbara County, California, and found them to be quite heavily contaminated with the spores of Clostridium botulinum, type A.

However, by far the most extensive survey recorded along this line of investigation is that of K. F. Meyer and B. J. Dubovsky[8] in which they examined 1,538 samples of soil, vegetables, feed and manure from every state in the United States, except Virginia, in an attempt to prove that the spores of this anaerobe are widely distributed throughout the United States. Because a majority of the recognized outbreaks of "botulinus poisoning" have occurred in California and along the Pacific coast many persons have come to think of that region as an epidemic focus of botulism, and it was with the idea of demonstrating that

this was not alone a problem confined to the Western coast states that Meyer and Dubovsky began their work.

From their findings they conclude, "botulinus is a common soil anaerobe of the Western States of the Cordilleran system but is less frequently encountered in the Atlantic States, and is relatively rare in the Middle States, the Great Plains and the Mississippi Valley." They found the soils which were subjected to more intensive cultivation and fertilization contain, as a rule, Clostridium botulinum type B. While type A was predominant in virgin soil. Both as a result of these findings and of those in their survey of California alone (A) they believe it very probable that type B is an adaptation mutant to the physical and chemical influences encountered by type A in tilled and cultivated soil; that type A has its origin in the virgin soil of the mountain ranges and as such is swept down into the valleys where, due to the change in physical and chemical conditions it becomes type B.

From the State of Kansas, K. F. Meyer and B. J. Dubovsky[7] collected: 10 samples of soil (horse corral, cow pastures, pig pen, grain fields, etc.) and 20 specimens of vegetables, corn stalks, alfalfa, bean stalks, and lettuce. From this material only two, or six and six-tenths per cent positive cultures were obtained. Both of these, one a sample of beets and one of string beans, were bought on the market, and both contained Clostridium botulinum, type A. All the soil samples gave negative results. In this same

survey, two human outbreaks in Colorado were found to be caused by commercially canned string beans or spinach grown and packed in the state of Kansas.

Recently, Freda M. Backman and Edith Haynes[11] in making an independent survey of the occurrance of toxin producing anaerobes in Wisconsin found no evidence of strong toxin producing botulinus in the soils of the state.

It was in an attempt to carry further the work done on Kansas soils by Meyer and Dubovsky that the following investigations were started. In view of the fact that Clostridium botulinum had been found in the soils of neighboring states, it seemed reasonable to believe a more extensive survey of the soils in this state might reveal the presence of that organism.

After approximately 100 samples had been examined and none had shown any evidence of containing either type A or type B toxin, the question was raised as to whether or not the results might be due to the unfavorable chemical constituents of different types of soil. Perhaps there were some soil samples tested that contained Clostridium botulinum, but the percentage of acids or alkalis present inhibited the production of toxin so that it was not detected. Jacques J. Bronfenbrenner and Monroe J. Schlesinger[12] in a recent publication report that in a weakly alkaline medium the potency of botulinus toxin is reduced one-tenth in 24 hours. Konrad Schubel[13] noticed attenuation of the toxin in N/4 HCl while in 1904 Landmann[14] found the toxin resis-

ted exposure to N/5 HCl for 24 hours. In 1922, Bronfenbrenner and Schlesinger[15] reported that the toxicity of the Clostrium botulinum toxins might be greatly increased by an acidification of approximately pH 4.0. Contrary to this, Geiger and Gouwens[16] found no increase in the potency of the toxin at any hydrogen ion concentration used in their experiment regardless of the length of time exposed. In this same connection, Freda M. Backman's experiments[9] show that the organism grows and produces toxin in fermented vegetables, sauerkraut, and green and yellow beans. J. B. Cutter[10] reports two deaths resulting from the consumption of tomato chili sauce, a food product which is more or less acid. However, contradictory the reports are on the effects of acids on botulinus toxin, it seems to be agreed by Bronfenbrenner, Scheslinger and Schubel[11,12] that the toxin appears to be less resistant to alkalis than to acids. In view of these findings and the fact that similar experiments have never, to my knowledge, been reported, it seemed worth while to test the most commonly found types of soil in Kansas for any inhibitory effects their chemical constituents might have on toxin production.

Briefly state, the purpose of the present investigation were:

1. To find to what extent Clostridium botulinum is present in the soils of Kansas.

2. To discover whether or not the organism is able to

produce a strong toxin equally well in the different types of soils common in Kansas, or if some might not inhibit toxin production altogether.

3. If Clostridium botulinum does occur in the soils of this state, to discover which type is present to the greater extent, type A or B.

## EXPERIMENTAL.

### Collection and Treatment of Samples.

Soil specimen of approximately 200 - 300 grams each, were collected in carefully autoclaved glass stoppered bottles. The stoppers were made secure by covering with squares of cloth held in place by a rubber band. Two of these were sent to each locality tested with directions to fill, one with soil from timber land or a meadow (soil known not to have been recently cultivated) and the other with cultivated soil preferably from a bean or corn patch.

When these samples reached the laboratory, they were measured out in 20 gram lots and placed in freshly autoclaved centrifuge tubes containing 10 c.c. of sterile physiological salt solution. To destroy the non-spore bearing organisms, these suspensions were heated to 70° C for two hours after which they were mixed with 50 c.c. oil stratificed beef heart media and allowed to incubate at 37° C for ten days. Cotton plugs were used for stoppers to the flasks and the mouth of the flask was covered over with sealing wax to prevent any air from entering.

The veal heart media used has been described by Burke[17] and was made as follows: Fresh veal hearts were freed of all fat and chopped up finely in a meat grinder. To this was then added two volumes of water and the mixture brough slowly to the boiling point. It was next placed in 100 c.c. hard glass flasks, 50 c.c. to a flask, and sterilized at 15 pounds for thirty minutes. (After a few trials, it was found

necessary to autoclave the media a little longer than is customary because of the rather large particles of meat present). Adjustment to pH 7.2 to 7.4 was made as the media was used.

## Identification of Toxin.

For the purpose of separating the toxic from the non-toxic cultures, the following procedure was followed after the cultures had incubated from ten to thirteen days:

Ten cubic centimeters of the supernatent fluid was carefully removed without stirring up the meat and soil sediment in the bottom of the flask. This material was placed in a sterile tube which was stoppered with a cotton plug held in place by means of a rubber band, and centrifugalized at high speed for one hour. The supernatent fluid was then removed to another sterile test tube. This perfectly clear fluid supposedly contained only the toxin since the organisms were separated out by centrifugalization. Two cubic centimeters of this fluid were inoculated either subcutaneously or intraperitoneally on the median abdominal line of a guinea pig. The guinea pigs used were from healthy stock in order to avoid symptoms or death from an intercurrent infection, and a fresh pig was used for each sample tested. B. J. Dubovsky and K. F. Meyer[19] state that in 90% of their tests elimination of non-toxic samples was possible by this procedure.

In connection with the type of injections used in this work, it is interesting to note the recent report of Paul

J. Hanzlik and Howard T. Karsner[18] who found that intraperitoneal injections of a variety of substances in guinea pigs caused definite anaphylactoid phenomena, inclusive of the pulmonary changes, fatalities and in some instances, inhibition or prolongation of blood coagulation. However, kaolin injected in this manner did not bring about marked anaphylactoid symptoms, only slight dyspnoea, but did produce similar histological changes in the lungs.

In cases where the pigs succumbed within 8 days, it was next necessary to determine approximately the strength of the toxin injected before antitoxin neutralization could be tried. This was done by injecting 1 c.c. of varying dilutions of the toxin and taking the largest dilution that killed a 250 gram guinea pig in 96 hours as that which most nearly contained one minimal lethal dose of the toxin. (Table I).

TABLE I.

| Specimen | Dilution injected in 1 c.c. amounts | Time required to kill 250 gm. pig |
|---|---|---|
| Lab. Strain | 1:50 | 9 hours |
| " " | 1:100 | 12 hours |
| " " | 1:500 | 24 hours |
| " " | 1:1000 | 74 hours |
| " " | 1:2000 | Recovered |

In the above example, 1 c.c. of the undiluted toxin would be considered as containing approximately 1000 minimal

lethal doses.

For routine toxin-antitoxin tests, three 250 gram guinea pigs were injected intraperitoneally as follows:

TABLE II.

| The toxins and anti-toxins for this test were mixed and allowed to remain at room temperature for from one to two hours before injection | | |
|---|---|---|
| Guinea Pig No. | Supernatant Culture Fluid. | Antitoxin. |
| 1 | Two c.c. supernatant fluid which contains less than 1000 MLD per c.c. | More than two units of antitoxin for Clostridium botulinum, type A. |
| 2 | Two c.c. supernatant fluid which contains less than 100 MLD per c.c. | More than two units of antitoxin for Clostridium botulinum, type B. |
| 3 | Two c.c. supernatant fluid which contains less than 1000 MLD per c.c. | Control no Antitoxin. |

There are two distinct types of Clostridium botulinum antitoxin on the market, known as types A and B. Each antitoxin is specific for the type of toxin it is prepared against and it will not protect against the heterologous toxin. For this reason when the type of botulinus is unknown, it is necessary to use both types of antitoxin for protection. If the material contains the toxin of Clostri-

dium botulinum type A the pig receiving no antitoxin and that receiving type B antitoxin will die, while that receiving type A antitoxin will survive. Similarily if the soil contains type B toxin only that pig receiving the type B antiserum will live.

Dubovsky and Meyer[19] state that toxin-antitoxin tests have been successful with cultures containing 1 to 10 minimal lethal doses per 2 c.c. Gertrude D. Burke[2] found that "a reliable toxin-antitoxin test may be obtained with a toxin, 1 c.c. of which requires four days to kill. With a weaker toxin, the antitoxin test is not reliable for in some cases the pigs develop typical symptoms of botulism but recover after about two weeks while in other cases they remain well for a month and then die with typical symptoms after a few days illness."

Isolation of Organisms.

In an attempt to isolate organisms from the two or three cultures which proved toxic to guinea pigs, three methods for the isolation of anaerobic bacteria were tried. The third and fourth methods were the same with the exception of the procedure for fishing. Because soil is very rich in both facultative anaerobes and strict anaerobes, it was found necessary first to make serial dilutions of the original cultures in the case of all three methods.

The first method to be tried involved the principle of the absorption of oxygen[20]. Dry pyrogallic acid (10 grams per litre of air space) was placed in the bottom of an or-

dinary chemical desiccator, and 15 c.c. N/10 NaOH was poured on the pyrogallic acid. Plain agar seeded with varying dilutions of the organisms was poured into sterile petri dishes and allowed to cool. The petri dishes were then placed in the desiccator and the jar closed at once. This method was abandoned after a time because of the large number of plates and the scarcity of desiccators. Then, too, because of the length of time required for the appearance of organisms by this procedure both of the other methods are to be preferred to this one.

The technique of the second and third methods are as follows: A series of deep agar shake cultures were made from the beef heart media. Just before the inoculations were made, the agar was boiled for 15 minutes and cooled to approximately 45° C. As soon as the inoculation was made, the agar was shaken well then allowed to cool. In practically every case, colonies appeared in 24 hours at 37° C.

For fishing, a method described by Burke[17] was next tried. The cotton plug was removed from the culture tube and the tube inverted, with the open end pointed downward into a sterile test tube of slightly larger diameter. The closed end of the tube containing the agar culture was then passed into the flame of a bunsen burner. The expansion, due to the heat, drove the agar cylinder out of the culture tube into larger tube, from which it was emptied into a petri dish for fishing.

The second procedure for fishing was used by Earnest C.

Dickson and Georgina E. Burke[21]. The culture tubes were filed and broken at a point slightly below the desired colony and the agar column forced out until the colony was just below the broken rim of the tube. The exposed surface was sterilized by means of a heated platinum wire. Colonies were fished through this sterilized surface.

In both of these methods for fishing, it was sometimes necessary to use a hand lens in examining the agar tube for desirable colonies to pick. In order to get sufficient separation of the colonies to allow for observation and isolation, it is very essential that the agar shakes be thinly seeded.

If the colony chosen for picking was located near the bottom of the tube, the second method of fishing was found to be more desirable than the first for the reason that the heating of the tube to force the column out into the second tube sometimes melts a portion of the agar in the butt of the tube thus destroying any colonies growing there. With this exception, these two methods were both very satisfactory.

## Results.

It is impossible to make an exhaustive survey of this nature for obvious reasons. In this part of the experiment, 131 samples of soil were tested representing some 46 different counties. The distribution of these specimens may be seen in Chart I. The results of tests with material from various counties are recorded in Table III.

1. Cheyenne
2. Sherman
3. Wallace
4. Greeley
5. Hamilton
6. Stanton
7. Morton
8. Stevens
9. Grant
10. Kearny
11. Wichita
12. Logan
13. Thomas
14. Rawlins
15. Decatur
16. Sheridan
17. Gove
18. Scott
19. Lane
20. Finney
21. Haskell
22. Gray
23. Seward
24. Meade
25. Clark
26. Ford
27. Hodgeman
28. Ness
29. Trego
30. Graham
31. Norton
32. Phillips
33. Rooks
34. Ellis
35. Rush
36. Pawnee
37. Edwards
38. Kiowa
39. Comanche
40. Barber
41. Pratt
42. Stafford
43. Barton
44. Russell
45. Osborne
46. Smith
47. Jewell
48. Mitchell
49. Lincoln
50. Ellsworth
51. Rice
52. Reno
53. Kingman
54. Harper
55. Sumner
56. Sedgewick
57. Harvey
58. McPherson
59. Saline
60. Ottawa
61. Cloud
62. Republic
63. Washington
64. Clay
65. Saline
66. Marion
67. Butler
68. Cowley
69. Chautauqua
70. Elk
71. Greenwood
72. Chase
73. Morris
74. Geary
75. Riley
76. Marshall
77. Pottawatomie
78. Wabaunsee
79. Lyon
80. Nemaha
81. Jackson
82. Shawnee
83. Osage
84. Coffey
85. Woodson
86. Wilson
87. Montgomery
88. Labette
89. Neosho
90. Allen
91. Anderson
92. Franklin
93. Douglas
94. Jefferson
95. Atchison
96. Brown
97. Doniphon
98. Leavenworth
99. Wyandotte
100. Johnson
101. Miami
102. Linn
103. Bourbon
104. Crawford
105. Cherokee

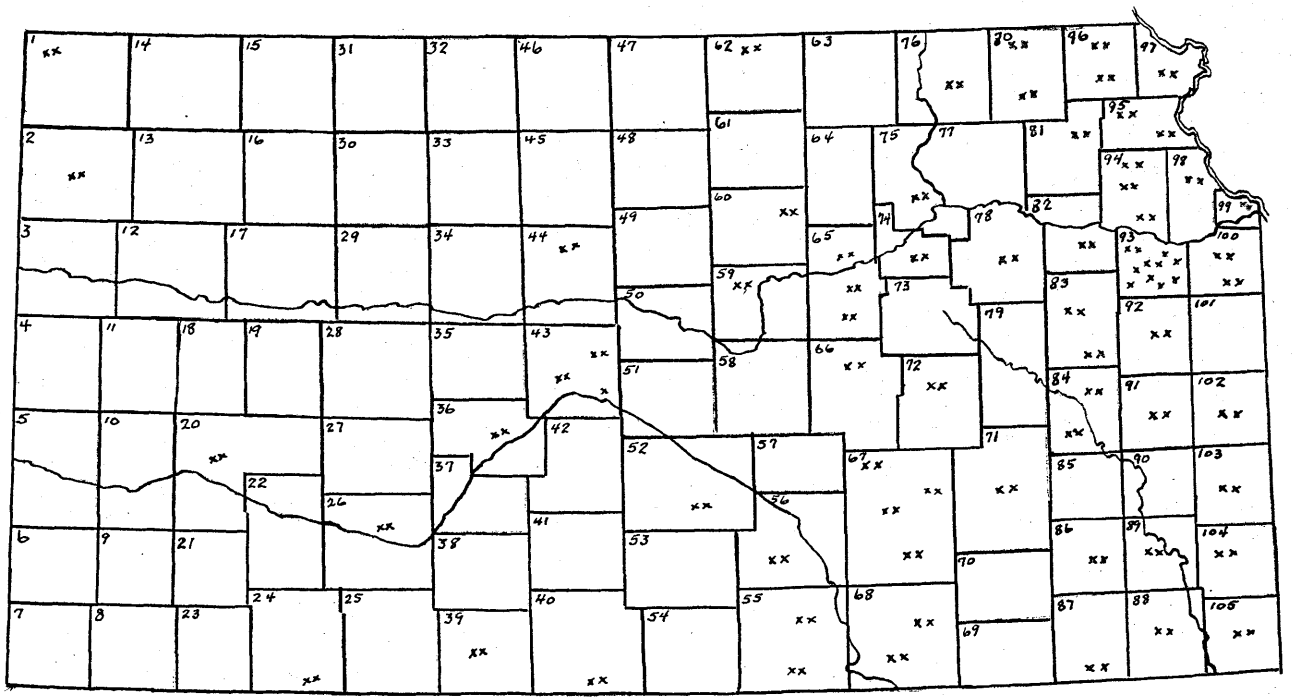

CHART I.

Map of Kansas Showing the Distribution of the Samples of Soil Examined in This Survey.

TABLE III.

Summary of Laboratory Findings.

| No. | County | # of Samples | Character of Specimen | Incubation Time | Toxic to Guinea Pigs. |
|---|---|---|---|---|---|
| 1 | Anderson | 1 | Cultivated | 10 days | Survived |
| 2 | Anderson | 1 | Uncultivated | 10 days | Survived |
| 3 | Atchison | 2 | Cultivated | 10 days | Survived |
| 4 | Atchison | 2 | Uncultivated | 10 days | Survived |
| 5 | Barber | 1 | Cultivated | 10 days | Died in 48 hours |
| 6 | Barber | 1 | Uncultivated | 10 days | Survived |
| 7 | Barton | 3 | Cultivated | 12 days | Survived |
| 8 | Barton | 3 | Uncultivated | 12 days | Survived |
| 9 | Bourbon | 1 | Cultivated | 12 days | Survived |
| 10 | Bourbon | 1 | Uncultivated | 12 days | Survived |
| 11 | Brown | 2 | Cultivated | 11 days | Died in 18 hours |
| 12 | Brown | 2 | Uncultivated | 11 days | Survived |
| 13 | Butler | 4 | Cultivated | 11 days | Survived |
| 14 | Butler | 4 | Uncultivated | 11 days | Survived |
| 15 | Chase | 1 | Cultivated | 13 days | Survived |
| 16 | Chase | 1 | Uncultivated | 13 days | Survived |
| 17 | Cherokee | 1 | Cultivated | 10 days | Survived |
| 18 | Cherokee | 1 | Uncultivated | 10 days | Survived |
| 19 | Cheyenne | 1 | Cultivated | 10 days | Survived |
| 20 | Cheyenne | 1 | Uncultivated | 10 days | Survived |
| 21 | Clay | 1 | Cultivated | 10 days | Survived |
| 22 | Clay | 1 | Uncultivated | 10 days | Survived |
| 23 | Comanche | 1 | Cultivated | 10 days | Survived |
| 24 | Comanche | 1 | Uncultivated | 10 days | Survived |
| 25 | Coffey | 2 | Cultivated | 13 days | Survived |
| 26 | Coffey | 2 | Uncultivated | 13 days | Survived |
| 27 | Cowley | 2 | Cultivated | 10 days | Survived |
| 28 | Cowley | 2 | Uncultivated | 10 days | Survived |
| 29 | Crawford | 1 | Cultivated | 13 days | Survived |
| 30 | Crawford | 1 | Uncultivated | 13 days | Survived |
| 31 | Dickinson | 3 | Cultivated | 10 days | Survived |
| 32 | Dickinson | 3 | Uncultivated | 10 days | Survived |
| 33 | Doniphan | 1 | Cultivated | 13 days | Survived |
| 34 | Doniphan | 1 | Uncultivated | 13 days | Survived |
| 35 | Douglas | 3 | Cultivated | 10 days | Survived |
| 36 | Douglas | 8 | Uncultivated | 10 days | Survived |
| 37 | Finney | 1 | Cultivated | 13 days | Survived |
| 38 | Finney | 1 | Uncultivated | 13 days | Survived |
| 39 | Franklin | 1 | Cultivated | 13 days | Survived |
| 40 | Franklin | 1 | Uncultivated | 13 days | Survived |
| 41 | Ford | 1 | Cultivated | 10 days | Survived |
| 42 | Ford | 1 | Uncultivated | 10 days | Survived |

TABLE III.

(Continued)

| No. | County | # of Sample. | Character of Specimen | Incubation Time | Toxic to Guinea Pigs. |
|---|---|---|---|---|---|
| 43 | Geary | 1 | Cultivated | 10 days | Survived |
| 44 | Geary | 1 | Uncultivated | 10 days | Survived |
| 45 | Jefferson | 3 | Cultivated | 12 days | Survived |
| 46 | Jefferson | 3 | Uncultivated | 12 days | Survived |
| 47 | Johnson | 2 | Cultivated | 10 days | Survived |
| 48 | Johnson | 2 | Uncultivated | 10 days | Survived |
| 49 | Labette | 1 | Cultivated | 10 days | Survived |
| 50 | Labette | 1 | Uncultivated | 10 days | Survived |
| 51 | Leavenworth | 1 | Cultivated | 13 days | Survived |
| 52 | Leavenworth | 1 | Uncultivated | 13 days | Died in 5 hours. |
| 53 | Linn | 1 | Cultivated | 12 days | Survived |
| 54 | Linn | 1 | Uncultivated | 12 days | Survived |
| 55 | Marion | 1 | Cultivated | 10 days | Survived |
| 56 | Marion | 1 | Uncultivated | 10 days | Survived |
| 57 | Marshall | 1 | Cultivated | 10 days | Survived |
| 58 | Marshall | 1 | Uncultivated | 10 days | Survived |
| 59 | Meade | 1 | Cultivated | 10 days | Survived |
| 60 | Meade | 1 | Uncultivated | 10 days | Survived |
| 61 | Montgomery | 2 | Cultivated | 13 days | Survived |
| 62 | Montgomery | 2 | Uncultivated | 13 days | Survived |
| 63 | Nemaha | 1 | Cultivated | 10 days | Survived |
| 64 | Nemaha | 1 | Uncultivated | 10 days | Survived |
| 65 | Neosho | 1 | Cultivated | 13 days | Survived |
| 66 | Neosho | 1 | Uncultivated | 13 days | Survived |
| 67 | Osage | 1 | Cultivated | 10 days | Survived |
| 68 | Osage | 1 | Uncultivated | 10 days | Survived |
| 69 | Ottawa | 1 | Cultivated | 12 days | Survived |
| 70 | Ottawa | 1 | Uncultivated | 12 days | Survived |
| 71 | Pawnee | 1 | Cultivated | 10 days | Survived |
| 72 | Pawnee | 1 | Uncultivated | 10 days | Survived |
| 73 | Reno | 1 | Cultivated | 13 days | Survived |
| 74 | Reno | 1 | Uncultivated | 13 days | Survived |
| 75 | Russell | 1 | Cultivated | 10 days | Survived |
| 76 | Russell | 1 | Uncultivated | 10 days | Survived |
| 77 | Saline | 1 | Cultivated | 10 days | Survived |
| 78 | Saline | 1 | Uncultivated | 10 days | Survived |
| 79 | Seagwick | 1 | Cultivated | 10 days | Survived |
| 80 | Seagwick | 1 | Uncultivated | 10 days | Survived |
| 81 | Shawnee | 1 | Cultivated | 12 days | Survived |
| 82 | Shawnee | 1 | Uncultivated | 12 days | Survived |

| No. | | # of Sample | Character of Specimen | Incubation Time. | Toxic to Guinea Pigs. |
|---|---|---|---|---|---|
| 83 | Sherman | 1 | Cultivated | 10 days | Died in 8 hours. |
| 84 | Sherman | 1 | Uncultivated | 10 days | Died in 10 hours. |
| 85 | Sumner | 1 | Cultivated | 10 days | Survived |
| 86 | Sumner | 1 | Uncultivated | 10 days | Survived |
| 87 | Wabaunsee | 1 | Cultivated | 10 days | Survived |
| 88 | Wabaunsee | 1 | Uncultivated | 10 days | Survived |
| 89 | Wilson | 1 | Cultivated | 13 days | Survived |
| 90 | Wilson | 1 | Uncultivated | 13 days | Survived |
| 91 | Wyandotte | 1 | Cultivated | 13 days | Survived |
| 92 | Wyandotte | 1 | Uncultivated | 13 days | Survived |

Out of the 131 samples run, there were only five fatalities and these pigs showed no symptoms other than dyspnoea, rather slight at first but gradually growing worse until death, and a general weakening of the whole system. There was never at any time any paralysis that could be observed. Pigs lingered from one-half to three hours after the first symptoms appeared until they finally died. Toxin-antitoxin tests were run on Brown County cultivated, Sherman County cultivated and uncultivated, Barbar County cultivated, and Leavenworth County uncultivated with the following results:

TABLE IV.

| Specimen | Supernatant Fluid MLD'S per c.c. | Antitoxin | Results on 250 Gram Guinea Pig. |
|---|---|---|---|
| Barber County | 2 c.c. (50) | Control, no antitoxin | Sick, but recovered |
| | 2 c.c. (50) | 2 units antitoxin Type A | No reaction |
| | 2 c.c. (50) | 2 units antitoxin Type B | No reaction |
| Brown Co. | 2 c.c. (less than 50) | Control, no antitoxin | Sick, but recovered |
| | 2 c.c. (less than 50) | 2 units antitoxin, type A | No reaction |
| | 2 c.c. (less than 50) | 2 units antitoxin, type B | No reaction |
| Leavenworth County | 2 c.c. (less than 1000) | Control, no antitoxin | Died in 9 hrs. Dyspnoea, Dizzy, Greatly weakened, Tried to climb out of cage |
| | 2 c.c. (less than 1000) | 2 units antitoxin, type A | Died in 10 hours, Displayed same symptoms as control pig |
| | 2 c.c. (less than 1000) | 2 units antitoxin, type B | Died in 9 hrs. Displayed same symptoms as control pig |
| Sherman County Cultivated | 2 c.c. (less than 1000) | Control, no antitoxin | Died in 10 hrs. Dyspnoea only symptom. |
| | 2 c.c. (less than 1000) | 2 units antitoxin Type A | Survived |
| | 2 c.c. (less than 1000 | 2 units antitoxin, type B | Survived |
| Sherman County Uncultivated. | 2 c.c. (less than 1000) | Control, no antitoxin | Died in 10 hrs. Dyspnoea only symptom |
| | 2 c.c. (less than 1000) | 2 units antitoxin, type A | Survived |
| | 2 c.c. (less than 1000) | 2 units antitoxin, type B | Survived |

From these results, it was concluded that any toxins c contained in the samples from Barber and Brown counties, if botulinus toxin at all, were too weak to identify by this method. From Brown county, a gram positive organism having a central spore was isolated. This organism grew rather slowly under aerobic conditions so was not a strict anaerobe. On the other hand, the organism isolated from Barber County was an obligatory anaerobe, large, gram positive and contained an excentric spore which looked not unlike Clostridium botulinum.

According to the results of the antitoxin neutralization test, Jefferson county uncultivated did not contain Clostridium botulinum of either type, since neither of the antitoxins protected. Sherman county cultivated and uncultivated appeared to contain both types A and B toxins. A large gram positive spore bearing organism having an ecentric spore was isolated from both cultures.

As a result of the large number of negative findings, it was decided to run checks on the most representative types of soils in Kansas to see if any of these under the same conditions and treatment of those samples collected from the various counties inhibited the production of Clostridium botulinum toxin. As there are easily over 1000 different types of soil in the state, it was impossible to run every type, but through the kindness of R. I. Throckmorton, professor of Soils at the Kansas State Agricultural College, twelve sam-

ples were obtained which he considered represented the most widely distributed varieties. Listing the counties from which they were obtained, their origin and the type, they are:

| County | Origin | Type |
|---|---|---|
| Cherokee | shale | Cherokee Silt Loam |
| Greenwood | limestone | Crawford Silt Loam |
| Cherokee | sandstone shale | Bates Silt Loam |
| Greenwood | sandstone | Boone Sandy Loam |
| Cherokee | sandstone shale | Summitt Silt Loam |
| Shawnee | mostly sandstone and shale | Oswego Silt Loam (Gumbo) |
| Shawnee | glacial | Shelby Silt Loam |
| Jewell | wind | Colby Silt Loam |
| Greenwood | cherty limestone | Crawford Gravely Loam |
| Shawnee | alluvial | Osage Silt Loam |
| +Jewell | alluvial | Lincoln Silt Loam |
| #Reno | alluvial | Arkansas Sandy Loam |

+Northwest part of state

#Southwest part of state

For seeding these samples, the following procedure was used: Broth cultures of Clostridium botulinum (obtained from Paul F. Orr of Harvard University) were centrifugalized and the sediment washed thoroughly with physiological salt solution to free it of any toxin which might be present. A

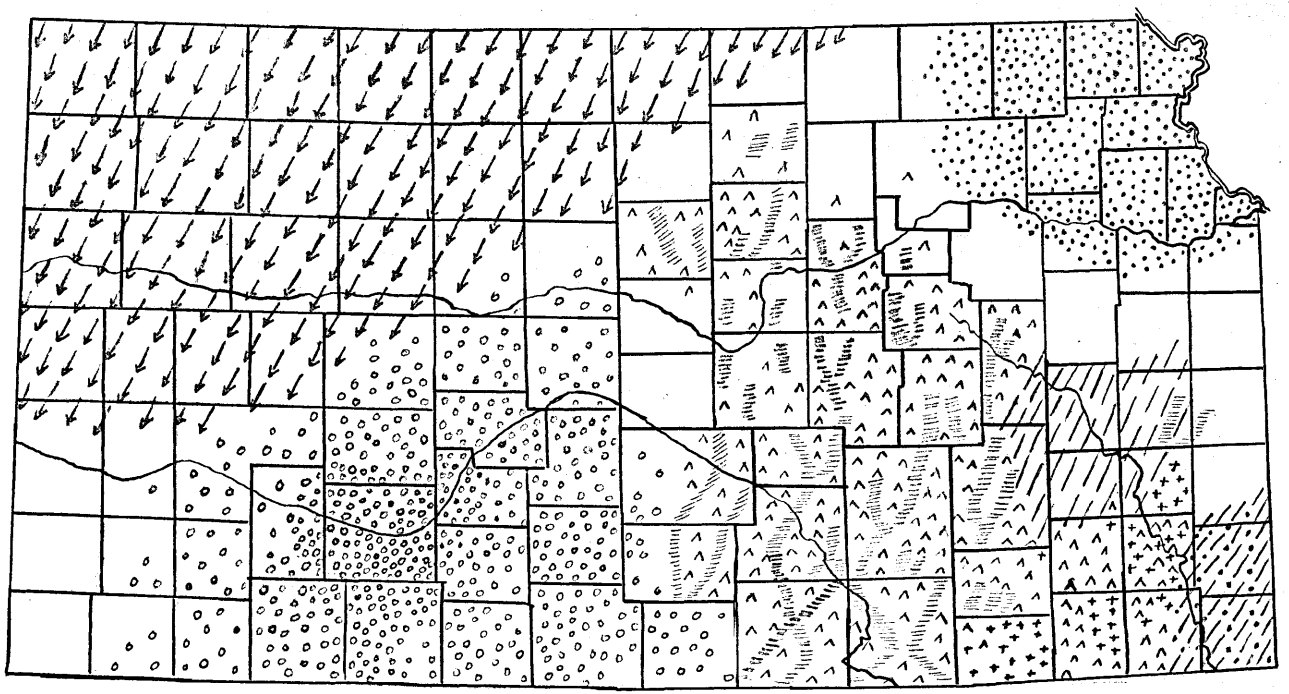

CHART II.

Map of Kansas Showing the General Distribution of the Different Types of Soil Used.

Wabash - glacial  Arkansas  Osage  Oswego

Lincoln and Colby  Cherokee  Boone  Summitt

suspension of the washed organisms was made by breaking up enough of the sediment in saline solution to make a slightly cloudy emulsion. One cubic centimeter was used for inoculating each 20 grams of soil. This contaminated material was then treated in the same manner as were the samples which came to the laboratory from over the state (page 7). Controls were run on each sample using soil that had not been seeded with Clostridium botulinum to rule out any soil that might be toxic in themselves. The results of this experiment may be noted in Table V.

TABLE V.

Soils Contaminated with Culture of Clostridium Bottulinum

| No. | Sample | Death to 250 Gram Guinea Pig | Symptoms |
|---|---|---|---|
| 1 | Shelby Silt Loam | 4 hours | Dyspnoea and general weakening of whole body. Died 1/2 hr. after first symptoms appeared. |
| 2 | Cherokee Silt Loam | 8 hours | Dyspnoea and general weakening of whole body. |
| 3 | Boone Sandy Loam | 12 hours | Same symptoms as above. |
| 4 | Crawford Gravely Loam | 7 hours | Same as above. |
| 5 | Summitt Silt Loam | 7 hours | Died 2 1/2 hrs. after first symptoms. |
| 6 | Bates Silt Loam | 12 hours | Same as above |
| 7 | Colby Silt Loam | 12 hours | Same as above |
| 8 | Lincoln Silt Loam | 10 hours | Same as above |
| 9 | Crawford Silt Loam | 12 hours | Same as above |
| 10 | Osage Silt Loam | 10 hours | Same as above |
| 11 | Arkansas Clay | 10 hours | Same as above |
| 12 | Oswego Silt Loam | 5 hours | Same as above |
| | Controls:- Samples of soil that were not seeded. | | |
| 13 | Shelby Silt Loam | 10 hours | Dyspnoea, greatly weakened. No paralysis that could be noted. |
| 14 | Cherokee Silt Loam | Survived | None |
| 15 | Boone Sandy Loam | 10 hours | Dypsnoea, greatly weakened. No paralysis. |

| No. | Sample | Death to 250 Gm. Guinea Pig | Symptoms |
|---|---|---|---|
| 16 | Crawford Gravely Loam | Survived | None |
| 17 | Summitt Silt Loam | Survived | None |
| 18 | Bates Silt Loam | Survived | None |
| 19 | Colby Silt Loam | Survived | None |
| 20 | Lincoln Silt Loam | Survived | None |
| 21 | Crawford Silt Loam | Survived | None |
| 22 | Osage Silt Loam | Survived | None |
| 23 | Arkansas Clay | Survived | None |
| 24 | Oswego Silt Loam | Survived | None |

In the controls (Table V), it may be noted that samples 1 and 3 along each brought about death of the guinea pig in less than 12 hours. One c.c. of serial dilutions was next injected into a number of pigs to determine approximately the minimal lethal dose and both samples were found to contain less than 50 MLD's per c.c. of undiluted culture fluid.

TABLE VI.

Results of the Toxin-antitoxin Test.

| Toxin | Anti-toxin | Amount injected (250 gram Guinea Pig) | Reaction |
|---|---|---|---|
| Shelby Silt Loam | Type A | .2 c.c. | Survived |
| Shelby Silt Loam | Type B | 2 c.c. | Survived |
| Shelby Silt Loam | Control | 2 c.c. | Pig died in 20 hrs. with typical symptoms. |
| Boone Sandy Loam | Type A | 2 c.c. | Survived |
| Boone Sandy Loam | Type B | 2 c.c. | Survived |
| Boone Sandy Loam | Control | 2 c.c. | Died in 7 hours Typical symptoms |

It was thought it might be of interest to find what effect the soils alone had on the reaction of the media; whether upon the additon of each of these twelve samples the media became acid or alkaline, for this might material-

ly effect the growth of any organisms contained. It may be seen by Table VII that the majority of the soils made the media decidedly more acid. Only in one case was the alkalinity increased.

TABLE VII.

Showing Change in Reaction of Supernatant Fluid Brought about by the Soils Alone.

| Sample | pH of Supernatant fluid before the Addition of the Soil. | pH of Supernatant Fluid 12 hrs. after Soil was Added (Ice box temperature). |
|---|---|---|
| Shelby Silt Loam | 6.8 | 5.0 - 5.2 |
| Cherokee Silt Loam | 6.8 | 5.2 |
| Boone Sandy Loam | 6.8 | 5.2 |
| Crawford Gravely Loam | 6.8 | 5.2 - 5.4 |
| Summitt Silt Loam | 6.8 | 5.2 - 5.4 |
| Bates Silt Loam | 6.8 | 5.2 - 5.4 |
| Colby Silt Loam | 6.8 | 6.2 |
| Lincoln Silt Loam | 6.8 | 6.6 |
| Crawford Silt Loam | 6.8 | 5.2 |
| Osage Silt Loam | 6.8 | 5.6 |
| Arkansas Clay | 6.8 | 7.2 |
| Oswego Silt Loam | 6.8 | 5.0 |

The soil types used in this experiment had been kept in air tight bottles for some twelve or thirteen years prior to this examination. The findings indicate the presence of Clostridium botulinum in two of the control samples but in view

of the fact that the stock bottles had not been sterilized before filling no conclusion can be drawn as to whether the organisms were in the soil when collected or whether they were contained in the bottles before the soil was introduced. Presumable Clostridium botulinum toxin was present otherwise the specific antitoxins would not have brought about protection to the guinea pigs, while the pigs not receiving the antitoxin died with the typical symptoms observed when injections were made with material known to have been contaminated.

## DISCUSSION.

The fact that both type A and type B antitoxins protected guinea pigs against the toxin contained in Shelby Silt Loam, Boone Sandy Loam, and Sherman cultivated and uncultivated, is somewhat puzzling. A possible explanation for this may be that both types of Clostridium botulinum toxins were present in the soil, the two together being in sufficient quantity to produce death to the guinea pigs, but each of them alone too dilute to be detected. This idea is further borne out by the fact that with the two together there were less than 50 minimal lethal doses per c.c. of culture media, which is a very small amount. It is of interest to note that Clostridium botulinum was able to survive and again produce toxin after being kept over a period of ten years in an air tight container.

Various methods of identifying Clostridium botulinum have been worked out in recent years. Aside from the relative simple antitoxin neutralization test described in this paper, Bronfenbrenner, Schlesinger and Calazans[22] in 1921 reported that Clostridium botulinum type A could be successfully differentiated from type B by means of seriologic tests. P. Schoenholz and K. F. Meyer[23] confirmed these findings but encountered occasional high cross-agglutinations. These two investigators found that by means of agglutinin tests that the B types could be divided into at least two groups, while the A types can be divided into three or four groups. Wm.

A. Starin and Gail M Dach[24] also demonstrated a distinct specificity of agglutinins for type A and B cultures. In 1923, R. A. Kelser [25] identified Clostridium botulinum both in pure and contaminated cultures by means of complement-fixation titrations.

In the work of this paper, there was no attempt made to introduce new methods of procedure. The reaction of media, amounts and preparation of field material to be examined, the period of incubation, and the identification of toxin have all been described by B. J. Dubovsky and K. F. Meyer[19].

Guinea pigs were chosen to be used in this work because of the comparatively large amounts of material that may be injected with safety. Paul F. Orr[26], Wagner, Dozier, Meyer[27] and others have successfully used mice for the identification of toxic material but in this case only 1 c.c. of the suspected material may be injected. If very weak toxins are encountered in the samples, they are only detected following the injection of at least 2 c.c., so for this reason mice would be unsuitable.

Graham, and Schwarze[28] introduced the feeding of chickens as a means of determining the type strain of Clostridium botulinum. Their observations show that Clostridium botulinum, type A, proved fatal when fed to mature chickens while type B, via the digestive tract was non-toxic to mature chickens. However, this procedure has been questioned by Paul F.

Orr[26] who found botulism could be produced in chickens with Type B as well as with Type A.

## CONCLUSIONS.

1. That strong toxin producing strains of Clostridium botulinum are not present to any extent in the soils of Kansas. Out of the 131 samples tested, both types A and B toxins appeared to be present in four samples of soil, but these were quite weak in comparison to what might be expected in view of the findings of other investigators.
2. That none of the different types of soil most commonly found in this state have any inhibatory effect on the production of Clostridium botulinum toxin. While none of these representative types appeared to inhibit, yet it might be possible to find soils from over the state that would, because of course the chemical composition of the same type of soil in different localities varies and the composition of all soils change from day to day. This is especially true of the surface soils.
3. Clostridium botulinum spores are able to protect the organism over a period of twelve or thirteen years in air tight vessels.

BIBLIOGRAPHY.

1. Van Ermengen.

Centralblatt fur Bakteriologie, Parasitenkunde u. Infectionskrankheiten. 1896, Band 19, p. 442.

2. Burke, Gertrude D.

Jr. Bact. 1919, Vol. 4, No. 5, p. 541.

3. Meyer, K. F. and Dubovsky, Bertha J.

Jr. Inf. Dis. 1922, Vol. 31, No. 6, p. 600.

4. Meyer, K. F. and Dubovsky, Bertha J.

Jr. Inf. Dis., Vol. 31, No. 6, p. 541.

5. Meyer, K. F. and Dubovsky, Bertha J.

Jr. Inf. Dis., Vol. 31, No. 6, p. 595.

6. Schoenholz, P., and Meyer, K. F.

Jr. Inf. Dis. 1922, Vol. 31, No. 6, p. 610.

7. Coleman, Geo. E.

Jr. Inf. Dis. 1922, Vol. 31, No. 6, p. 541.

8. Meyer, K. F. and Dubovsky, Bertha J.

Jr. Inf. Dis. 1922, Vol. 31, No. 6, p. 559.

9. Backman, Freda M.

Jr. Inf. Dis. 1924, Vol. 34, No. 2, p. 129.

10. Cutter, J. B.

Jr. Am. Med. Assoc. 1922, Vol. 70, No. 10, p. 825.

11. Backman, Freda M. and Haynes, Edith.

Jr. Inf. Dis. 1924, Vol. 34, No. 2, p. 132.

12. Bronfenbrenner, Jacques J. and Schlesinger, Monroe J.

Jr. Exper. Med. 1924, Vol. XXXIX, No. 4, p.

13. Schubel, Konrad.

Archive fur Experimentelle Pathologie und Pharmakologie, 1923, Bd. 96, Heft. 3/5, S. 193.

14. Landmann (Cited from Bronfenbrenner, Jacques J. and Schlesinger, Monroe J.

Jr. Exper. Med. 1924, Vol. XXXIX, No. 4.

15. Bronfenbrenner, Jacques J. and Schlesinger, M. J.

Jr. Amer. Med. Assoc., 1922, Vol. 78, p.

16. Geiger, J. C. and Gouwens, W. E. (Cited from an Editorial Amer. Med. Assoc. 1924, Vol. 82, No. 17, p. 1364.

17. Burke, G. S.

Jr. of Bact., Vol. 4, No. 5, p. 555, 1919.

18. Hanzlik, Paul J. and Karsner, Howard T.

Jr. Phar. and Exp. Ther., 1924, Vol. 23, No. 3, p. 243.

19. Dubovsky, B. J. and Meyer, K. F.

Jr. Inf. Dis. 1922, Vol. 31, No. 6, p. 501.

20. Jordan, Edwin O.

Text book of General Bacteriology, 6th Edition, p. 50.

21. Dixon, Earnest C., and Burke, Georgiana E.

Jr. Amer. Med. Assoc., 1918, Vol. 71, No. 7, p. 518.

22. Bronfenbrenner, Schlesinger, and Calazans.

(Cited from P. Schoenholz and K. F. Meyer, Jr. Inf. Dis., 1923, Vol. 32, No. 6, p. 420.

23. Schoenholz, P. and Meyer, K. F.
Jr. Inf. Dis., 1923, Vol. 32, No. 6, p. 517.

24. Starin, W. A. and Dack, Gail M.
Jr. Inf. Dis., 1923, Vol. 33, No. 2, p. 169.

25. Kelser, R. A.
Amer. Jr. of Pub. Health, Vol. 13, No. 5, p. 366, 1923.

26. Orr, Paul F.
Jr. Inf. Dis. 1921, Vol. 29, No. 3, p. 287.

27. Wagner, E., Dozier, C. C., and Meyer, K. F.
Jr. Inf. Dis., 1924, Vol. 34, No. 1, p. 63.

28. Grahm, Robert, and Schwarze, Herman.
Jr. Inf. Dis. 1921, Vol. 28, No. 4, p. 317.