CLOSTRIDIUM BOTULINUM

IV. RESISTANCE OF SPORES TO MOIST HEAT

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The several recent outbreaks of botulism, which have been traced to foods preserved by some method of heating, have focused critical attention on the thermal resistance of the spores of *Clostridium botulinum*. While several papers have been published on this subject, its importance justifies repetition of the work in order that the conclusions based on the data, may be as accurate as possible. The present investigation was initiated before some of the other papers were published and was continued even though the experiments repeated some of those already reported.

HISTORICAL

Two papers have been published by Weiss (1921a, 1921b) which bear directly on the subject under discussion. He reported that the free spores of *Clostridium botulinum* were destroyed within five hours at 100°C., within forty minutes at 105°C., and within six minutes at 120°C. Weiss believed that the spores were injured before death and that the death process was a gradual one. Such an opinion is in keeping with our knowledge of disinfection by moist heat. Young spores were more resistant than old ones. Those one month old were found to be many times more resistant than older ones. The hydrogen ion concentration was found to have the usual effect. In the second paper Weiss reported some data on the thermal resistance of spores in canned food liquors. The resistance, as would be expected, varied with the reaction of the food. In gooseberry juice the spores were killed in 30 minutes while about

180 minutes were required for killing the spores in lima bean juice. Burke (1919 a) reported that the spores could survive boiling for three hours or more. In another paper Burke (1919 b) reported that the spores grown in brain medium were more resistant than those grown in broth. Fifteen pounds pressure for ten minutes was believed to be insufficient for destroying the spores. Thom et al (1919) reported that the Boise strain withstood a pressure of 10 pounds for fifteen minutes or 100°C., for one hour. Fifteen pounds for fifteen minutes in the autoclave destroyed it.

EXPERIMENTAL

Spore suspensions and cultures. The cultures used in these determinations were obtained from Dr. Robert Graham, Department of Animal Pathology, University of Illinois. They were numbered as follows with source: 820-A, isolated from garbage which caused avian botulism in Urbana, type A organism; 854-A, also type A, obtained from the spleen of a calf which died showing all of the characteristic symptoms of botulism; 820-B, a type B organism, isolated from corn ensilage which was known to have caused botulism among cattle in Iroquois County; G-6-B, another type B strain, isolated from oat hay, which, when inoculated into monkeys, produced death with the characteristic symptoms. In producing the spores of *Clostridium botulinum* a brain medium of thinner consistency than that described by Weiss (1921a) was utilized.

Medium. This was made by adding one part of finely macerated sheep's brain to two parts of water. It was then sterilized in an autoclave at 15 pounds pressure for thirty minutes in 250 cc. Pyrex Erlenmeyer flasks, from 125 to 150 cc. of medium in each flask. This medium was used without adjustment to grow the organism and produce the spore suspension since it was found to be practically neutral (pH 6.8 to 7.0). Dickson and Burke reported the use of a medium consisting of a 1 per cent glucose peptic digest of liver broth adjusted to pH 7.3 to 7.5.

Inoculations. Inoculations were made by means of 10 cc. sterile pipettes, 10 cc. of a stock suspension in brain medium

being transferred to each flask. Melted paraffin was then poured into the flask which hardened to give a coating on top of about 1 cm. in thickness. At a later date we tried growing the organisms without covering the culture with paraffin to determine whether a larger number of spores could be produced in the suspension, but have not sufficient data at present to warrant any conclusions. This procedure has been suggested by various investigators for certain of the other pathogenic anaerobes.

The flasks were then incubated at room temperature for approximately 30 days in the dark. To determine the number of spores produced the Torrey anaerobic plate was used. The medium used was a 0.5 per cent glucose agar in which dilutions of 1 to 1000 and 1 to 10,000 were inoculated. A covering of glucose agar was then poured on top and finally a coating of paraffin, colored with Sudan III, as suggested by Northrup to make the colonies stand out in relief. These cultures were incubated from ten days to two weeks and counted. The average number of spores obtained from the dilution plate counts was 3,200,000 per cubic centimeter.

Apparatus. Thermal determinations were made using a DeKotinsky electric bath, with a thermo regulator attachment, turbine agitator, Nujol for the liquid of high boiling point, and Wasserman test tube rack for immersion of tubes. Temperatures of 100°C.; 105°C.; 110°C.; 115°C.; and 120°C; were used in the experimental heating of the organisms.

The cultures used were transferred by means of a sterile pipette from the 250 cc. flasks to sterile, special soft glass tubes (as recommended by Bigelow and Estey (1920), having an inside bore of 7 mm., an outside diameter of 9 mm., and 250 mm. in length), 1 cc. of media to each tube. The tubes were than drawn out, 70 mm. above the top of the medium, to obtain a short capillary neck above the medium. One set was then exhausted to give a vacuum of about 17 mm. and sealed at the capillary portion. The second set was not exhausted but left plugged with cotton and heated as open tubes.

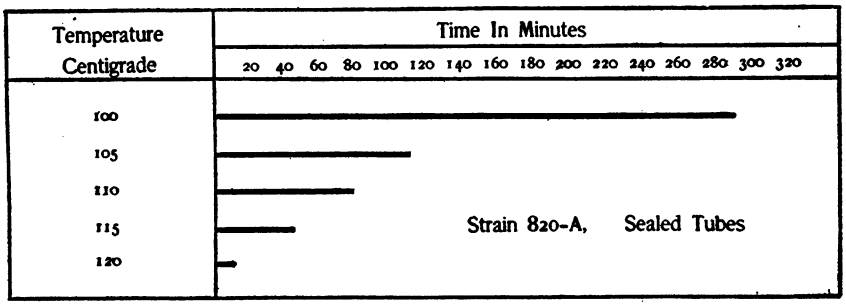
It was found to be troublesome to use the open tubes at temperatures above 100°C., since the medium boiled up in the tubes to the plugs and gave opportunity for too great an error. To

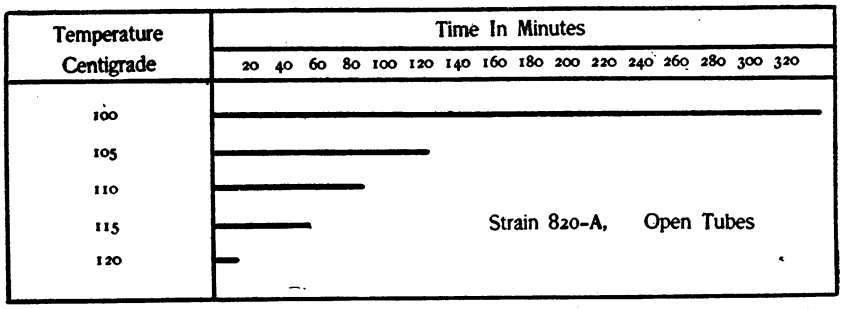
overcome this we drew out the tube in the same manner as for the sealed tubes, and pushed a tight cotton plug down until it was stopped by the capillary constriction. After heating, and immediately as each tube was taken from the bath, it was sealed in a flame to prevent any material which may have been unevenly heated from being drawn down into the tube as it cooled.

The spores used in these tubes were about thirty days old, it being impractical to utilize those exactly thirty days old at all times. The variation in resistances of a few days when the spores were approximately thirty days old was shown by Weiss to be negligible.

Incubation. One tube of each culture was taken out of the oil bath at stated intervals, cooled at once by immersion in cold water, and inoculated into 10 cc. tubes of sterile brain medium, care being taken to mix the spore suspensions with the brain medium. As stated above, the open tubes were sealed immediately and then cooled. The tubes of brain medium were then covered with paraffin to a depth of about 1.5 cm., plugged and incubated at room temperature in the dark. Owing to the time of completion of the thermal determinations, these tubes were incubated over the summer vacation and averaged an incubation period of four months.

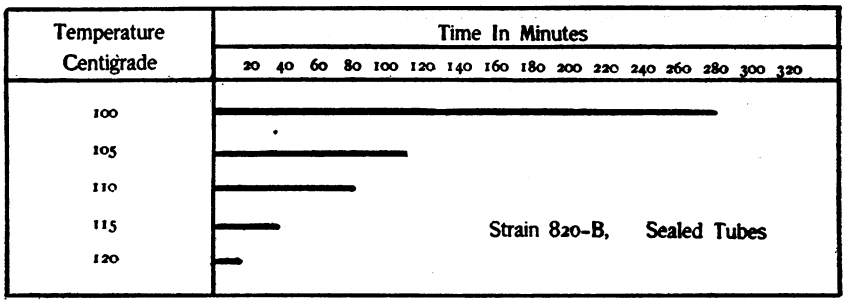
Inoculation. All cultures showing gas formation with the characteristic foul odor were discarded as indicative of active growth and the first one of the series showing no gas formation was taken for further study to ascertain if there had been any growth and toxin formation. One cubic centimeter of this latter culture was fed to a guinea pig through a sterile pipette, and the pig kept under observation for one month. If death with the characteristic symptoms did not ensue in this time the spores were considered to have been destroyed. It was found that our results supported the observations of Weiss, that the spores were progressively injured before death finally ensued. Some pigs inoculated orally, lived for periods of from three to nineteen days, showing that the organisms had been so injured that only a small amount of toxin or perhaps a toxin of low virulence had been produced.

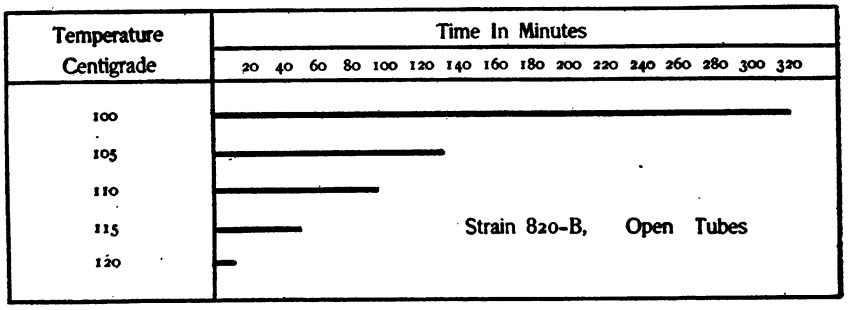




STRAIN 820-A

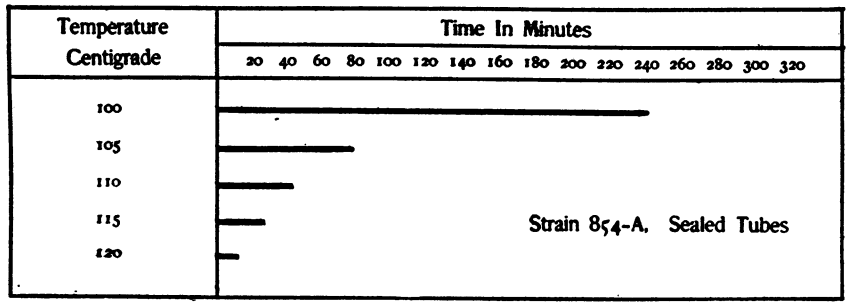
Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.

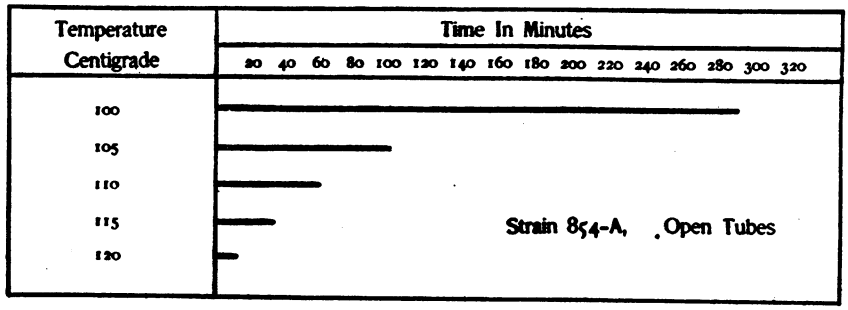




STRAIN 820-B

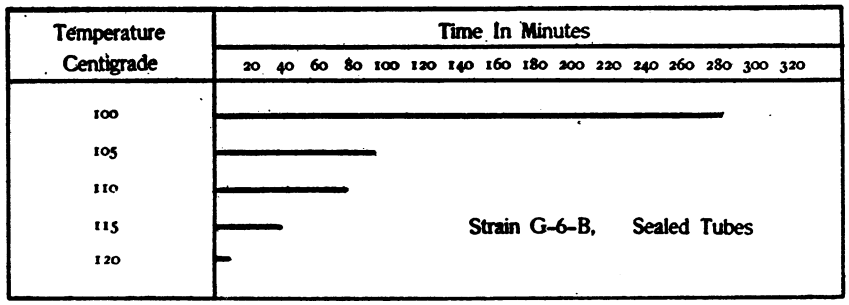
Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.

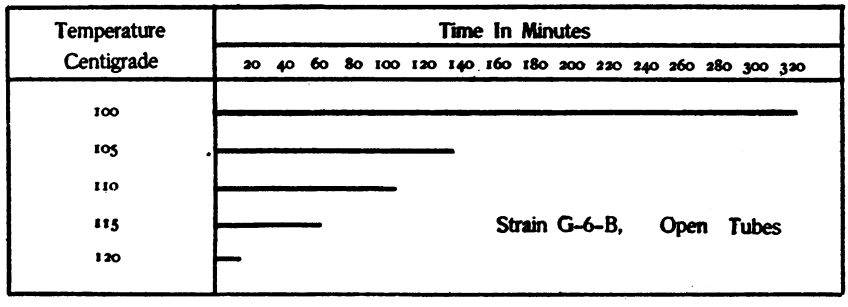




STRAIN 854-A

Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.





STRAIN G-6-B

Showing the time required in minutes for destroying the spores of *Clostridium botulinum* by moist heat.

DISCUSSION OF RESULTS

From the accompanying tables it will be seen that there is a variation in resistance of different strains of spores under controlled conditions. The most resistant spores were found to be destroyed in less than five hours at 100°C. in the cultures which had been exhausted to obtain a 17 mm. vacuum, and in five and one-half hours in the open tubes. Bigelow and Estey (1920) stated that the time necessary for the tubes to reach 100°C, after being immersed in the bath was on the average 15 seconds since the tubes used were small in diameter, only moderately thick, and completely immersed in the oil bath.

At 105° the cultures showed a variation of thirty minutes in death points obtained, the most resistant being destroyed in one hour and fifty minutes for the tubes exhausted to 17 mm. vacuum. In the open tubes there was also a variation of 30 minutes, the most resistant cultures being killed in two hours and ten minutes. The same conditions hold for 110°C., the cultures varying ten minutes and showing an irregularity in the final death point as determined by injection into guinea pigs. All cultures were found to be destroyed in less than an hour and a half in the sealed tubes. The open tubes showed a greater variation in thermal death time, the most resistant being killed in one hour and forty minutes, while the least resistant one was destroyed in fifty-five minutes. At 115°C. we see less variation in death point in vacuum tubes, all cultures being killed in forty minutes in the sealed tubes. The open tubes required a slightly longer time, the most resistant spores being destroyed in fifty minutes. At 120°C. no cultures survived ten minutes heating but 820-A does show slight toxin formation at the end of five minutes heating. This tube when inoculated into brain medium, incubated three months at 21°C., and fed to a guinea-pig, required nineteen days to cause death. All other cultures caused death of the guinea-pigs in twelve to seventy-two hours.

The variation between the open tubes and those exhausted and sealed to give a vacuum of 17 mm. is small but the time required in the case of the open tubes was always found to be longer.

The change in pH during the process of heating was found to be so small as to be negligible, the tubes having first been soaked over night in cleaning solution. The change in four hours with the tubes not so cleaned was found to average 0.6 by Morrison (unpublished data).

The reaction of our media was found by numerous determinations to average pH 6.8. This was kept in Pyrex flasks and used without adjustment. It was found that brain medium put up in soft glass flasks and kept for periods of twenty-one days or more had an average reaction of about pH 7.4. Either medium could be used to grow our cultures, both apparently being of equal value. With media testing pH 6.8, growth of the organism produced a gradual change to the alkaline side, reaching pH 7.2; with media at pH 7.4, instead of producing greater alkalinity, it, too, reached an average of 7.2. Weiss reports the pH as becoming stable at 7.5 after cultural growth in brain medium.

SUMMARY AND CONCLUSIONS

1. The spores of *Clostridium botulinum* in sealed tubes exhausted to 17 mm. vacuum are destroyed within a period of five hours at 100°C.; within two hours at 105°C.; within one and one-half hours at 110°C.; within forty minutes at 115°C.; and within ten minutes at 120°C.

2. A longer heating period was required to destroy spores of the same age in open tubes than in tubes exhausted and sealed, under the conditions that obtained in this investigation.

3. The death of spores is probably a gradual process. Surviving spores may have their internal mechanism so injured that weak toxins are formed.

REFERENCES

BIGELOW, W. D., AND ESTEY, J. R. 1920 Jour. Inf. Dis., **27**, 602.
BURKE, G. S. 1919a Jour. Amer. Med. Assoc., **78**, 88–92.
BURKE, G. S. 1919b Jour. Bact., **4**, 555–565.
THOM, C. ET AL. 1919 Jour. Amer. Med. Assoc., **73**, 907–912.
WEISS, H. 1921a Jour. Inf. Dis., **28**, 70–92.
WEISS, H. 1921b Jour. Inf. Dis., **29**, 362.