TOXIN FORMATION BY A VARIETY OF *B. BOTULINUS*
WHEN CULTIVATED AEROBICALLY UNDER VARIOUS
CONDITIONS: ITS POSSIBLE PRODUCTION IN THE ANIMAL BODY *

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Bacillus botulinus was first isolated by van Ermengem¹ who recovered it from meat that had produced an epidemic of botulism. He demonstrated that a soluble toxin secreted by the bacillus in the food was responsible for the condition and that the organism itself had no pathogenic power. That is to say, the bacillus was unable to produce the toxin under the conditions existing in the body.

The bacillus described by van Ermengem was a motile, sporulating anaerobe. This organism fermented dextrose, but did not coagulate milk. It required the presence of peptone in a neutral or slightly alkaline medium for toxin production. At temperatures above 30 C. toxin formation was inhibited. (This latter fact alone explained its lack of pathogenicity in animals having a temperature of 37 C.)

Although an obligatory anaerobe when in pure culture, van Ermengem and others showed that *B. botulinus* produced its toxin in the presence of oxygen when grown in symbiosis with certain aerobes.

Rabbits, cats, guinea-pigs, mice and monkeys are susceptible to the toxin. Chickens and frogs are not affected.

The symptoms produced are not those usually seen in a gastrointestinal intoxication. After an incubation period of from twenty-four to forty-eight hours there arise symptoms referable to the action of the toxin on the nervous system. In animals death is preceded by general motor paralysis and dyspnea, with secretion of mucus from the nose and mouth.

Postmortem, there is seen a general hyperemia of the organs with parenchymatous degeneration and minute hemorrhages. Amongst other changes, the muscles of the heart show signs of degeneration.

The work of van Ermengem has been confirmed in numerous instances. Recently there have been described in this country cases which vary somewhat from the original description.

*The latter part of this paper consists of preliminary notes not intended originally for publication. Further work along these lines has been postponed indefinitely, and for this reason it is thought best to publish what has been accomplished at the present time.

1. Kolle and Wassermann: Handbuch der path. Mikroorganismen, Ed. 2, 4:922.

Wilbur and Ophüls² reported an epidemic at Stanford University which apparently was caused by canned beans. That is to say, the toxin was produced in the absence of animal protein. Postmortem, they described a condition of thrombosis in the blood vessels at the base of the brain. In both of these points the epidemic varied from the original. No organism was isolated.

Later Dickson³ experimented with two strains of *B. botulinus*, one obtained from the New York Museum of Natural History, the other from Zinsser of Columbia. He concluded that "animal protein is not essential for the development of the toxin of botulism," that "an acid reaction of 3.2 per cent. to phenolphthalein does not prevent the formation of toxin," that "the toxin produces some disturbance of the circulatory system which leads to hyperemia and hemorrhages in the meninges and in the central nervous system, and to thrombosis in the arteries and veins of the meninges and of the central nervous system" and that "the lesions in the nerve cells are not due to a specific action of the toxin on the cell protoplasm, but they are secondary to disturbances of blood supply."

In another instance Nevin⁴ isolated from cottage cheese that had caused three deaths an organism closely resembling *B. botulinus* of van Ermengem. In this epidemic the clinical diagnosis was not made, and no organism capable of producing the symptoms was recovered postmortem.

The organism isolated from the cheese by Nevin was diagnosed as *B. botulinus*, but differed from this organism in the following particulars: It formed its toxin in a medium containing only the peptone of the meat, from which the medium was made, and it produced this toxin readily at 37 C.

These are very important variations from the organism of van Ermengem. Indeed, the production of toxin at 37 C. renders its nonpathogenicity a matter of doubt. Further, the fact that *B. botulinus* produces its toxin under aerobic conditions when cultivated with certain aerobes, and that according to Dickson³ a reaction of +3.2 does not entirely inhibit its production, and that animal protein is not essential, would indicate that toxin formation by *B. botulinus* might occur under conditions not generally thought possible.

EXPERIMENTAL

In the following experiments an attempt was made to study toxin production under unfavorable conditions such as might occur within and without the animal body in nature.

2. Wilbur and Ophüls: Archives Int. Med. **14**:589, 1914.

3. Dickson: Botulism, An Experimental Study, J. A. M. A. **65**:492, 1915.

4. Nevin, Mary: A Study of Cheese Causing Three Fatal Cases of Botulism. Read in abstract before the Conference of The American Public Health Association, Rochester, N. Y., Sept. 8, 1915.

The strains used by Dickson originally from Zinsser and the New York Museum of Natural History were found to have lost their toxin forming power as noted by Nevin. But the Nevin strain produced a powerful toxin and was used throughout the work.

The organism was found to differ somewhat from *B. botulinus* of van Ermengem in its behavior towards lactose. In mediums containing this sugar *B. botulinus* grows with difficulty, according to van Ermengem, who recommends dextrose as an enriching substance. The strain isolated by Nevin, on the contrary, grew very profusely in mediums containing from 1 to 2 per cent. lactose. In the hands of the writer it did not ferment this sugar with production of acidity or gas in spite of the fact that it possessed the power of acidifying and peptonizing milk. But according to Nevin it does possess this power.

In dextrose mediums it produced fermentation, as does *B. botulinus* of van Ermengem. The acidity of this reaction supposedly is not favorable to the toxin. Since growth without acidity occurred in the presence of lactose this substance was used instead of dextrose. Anaerobically, in sugar-free meat extract medium, the organism failed to grow.

One per cent. lactose agar of a neutral reaction was employed for the stock cultures and for testing the power of the organism to multiply under aerobic conditions. For toxin production it was cultivated in a broth adapted from that advocated by Rideal and Walker for testing disinfectants. This medium has the following ingredients:

	Gm. or c.c.
"Liebig's" extract of meat.....	20
Peptone (Witte's)	20
Salt	10
Distilled water	1,000

To this was added 20 gm. of lactose and the product was then made alkaline to the point of -0.5 . This medium will be referred to as L. R. W. broth.

Tests for Toxicity.—The toxicity of the Nevin organism was first tested against rabbits subcutaneously. In the preliminary experiment a very large dose was used. Two c.c. of a ten-day culture in L. R. W. broth grown anaerobically at about 30 C. in the dark were given by subcutaneous injection to a rabbit weighing 1,465 gm. Nineteen hours later the animal was found moribund, with all the muscles relaxed and saliva dribbling from the mouth. In another hour death occurred. The postmortem was performed immediately, and the following lesions were observed:

There was no gas in the tissues; serous exudate with some hemorrhage at the seat of inoculation; peritoneum and mesentery injected and organs, in general, hemorrhagic with the exception of the spleen, which appeared normal. The diaphragm and wall of the heart were likewise injected. Smears from the exudate, liver, spleen and heart's blood showed no organisms except for a few doubtful coli-like bacteria in the exudate. The organism was recovered in

pure culture by anaerobic methods from the heart's blood, liver and spleen. The culture from the heart's blood was retained and used in some of the experiments to be described, as it was believed that its toxin producing power might have been increased. It will be referred to as the H. strain.

The toxicity of the same strain when given per os was now tested. One c.c. of a four-day old culture grown as in the preceding experiment was placed on a little uncooked oatmeal, which completely absorbed the liquid. This preparation was given to a rabbit weighing 2,080 gm. The following morning the animal was observed to crawl instead of hopping in the usual fashion. No other symptom was noticed. On the second day the rabbit was unable either to hop or crawl, and died about fifty-four hours after eating the oatmeal. Postmortem the following points were observed:

There was marked injection subcutaneously, in mesentery, around the gut, on the stomach wall and in the diaphragm. The liver and spleen were congested and enlarged. The kidneys appeared normal. The bladder was full. Two lobes of the lungs were markedly congested. The heart was swollen and firm, and the vessels congested. There was no increase of fluid within the pericardium. Anaerobic cultures in L. R. W. broth from the heart, liver and spleen failed to show any growth.

One c.c. of the H. strain, made directly from the heart's blood of the first rabbit after three days' growth under similar anaerobic conditions, was next placed on some oats in a pan and given to a rabbit weighing 1,305 gm. The animal ate very little of the oats and since most of the culture trickled to the bottom of the pan only a very small portion of the broth could have been consumed. Yet on the morning following, the rabbit showed symptoms of weakness of the hind legs and lack of control of urine. In the afternoon of the same day the animal died, a little over twenty-four hours after ingestion of the poison. No postmortem was performed.

Finally, 0.1 c.c. of a five-day anaerobic culture of the original strain was placed in 1.0 c.c. of water, poured on a little oatmeal and given to a rabbit weighing 1,470 gm. The animal at first refused to eat this food, which was not consumed until the middle of the following day. No symptoms were apparent during the next twenty-four hours, but on the succeeding morning, about forty-five hours after the ingestion of the toxin, the rabbit appeared moribund, lying quietly on his side and dying soon.

The postmortem revealed marked injection of the blood vessels subcutaneously, around the gut, in the diaphragm, and on the heart wall. The spleen, liver and kidneys were markedly congested, but the lungs only moderately so. Slight hemorrhage was observed within the pericardium. Anaerobic and aerobic cultures were negative.

The importance of these experiments in which the toxin was given per os lay in the smallness of the lethal dose. For the toxin of *B. botulinus* of van Ermengem produces death under similar conditions only in doses of 5 to 10 c.c.

When tested against the cat the organism was not found to possess markedly toxic power, either per os or subcutaneously. One c.c. of a four-day anaerobic culture of the H. strain was given by stomach tube to a cat weighing 2,000 gm. The next day the animal's hind legs seemed a little stiff and in the afternoon it refused meat. The following morning a trembling in the tail was noticed, but in the afternoon the appetite returned, and from that time on no further symptoms were noticed.

To another cat was given a piece of meat in which had been injected 1 c.c. of a twelve-day anaerobic culture. This animal developed no symptoms.

A third cat was injected subcutaneously with 1 c.c. of a two-weeks-old culture of the same strain. On the following morning marked weakness in the hind legs was observed and the animal evidently preferred the dark. However, meat was not refused, and the symptoms gradually disappeared in the course of four days.

Another cat injected subcutaneously with 1 c.c. of a four-day anaerobic culture of the original strain failed to develop any symptoms during the first week, at the end of which period it was discarded.

The resistance of the cat to doses of 1.0 c.c. given subcutaneously differs from the reaction of the animal to the toxin produced by the strain of van Ermengem which caused death in quantities of from 0.1 to 1.0 c.c. But it should be noted that according to Nevin⁵ the toxin of the organism produced typical symptoms in kittens.

In regard to chickens and frogs, the organism of Nevin when fed per os acted according to the descriptions of *B. botulinus* of van Ermengem. In doses of 5 c.c. neither strain had any noticeable effect on two chickens fed with corn meal moistened with the cultures.

One frog was injected with 1.0 c.c. of the original strain, and another frog was placed in a jar containing 1.0 c.c. of the culture mixed with a little water. Neither developed any symptoms.

A third frog was placed in a jar containing 2 c.c. of the H. strain. It, likewise, failed to show any bad effects during a period of a week.

Finally⁶ the toxicity of the organism for horses when given per os was tested. For this purpose a four-day anaerobic culture of the H. strain in L. R. W. broth was used. Ten c.c. of this preparation were poured on some oats and given as feed to a young colt. Opportunity for close study of any symptoms was not available, but up to 5 a. m. of the second day after ingestion of the toxin, no symptoms were observed by the stable attendants. Three hours later, at 8 a. m., about forty-four hours after eating the oats, the animal was found dead. The postmortem was performed the same morning by Dr. Buckley and the following points noted:

Engorgement and inflammation of the jejunum and lungs; slight hemorrhages on the heart wall; engorgement of the lymph gland near the spleen and slight injection of the meninges. Transfers from the heart's blood, liver and spleen kept under anaerobic and aerobic conditions were sterile.

Experiments in Forage Poisoning.—The fatal result following the ingestion of the culture by the horse confirmed the possibility of the Nevin organism being the causative factor in forage poisoning and led to an attempt to reproduce this disease by treating forage with preparations cultivated under aerobic conditions similar to those found in nature. At the same time an effort was made to demonstrate the extent to which this organism might be cultivated in symbiosis with an aerobe in the presence of oxygen and still retain its toxin producing power.

Since the food supposed to be responsible for forage poisoning is frequently heavily seeded with yeasts, a variety of this species was chosen as symbiotic organism. It possessed the following characteristics:

Milk: Not changed.

Gelatin: Not liquefied.

Broth: Growth in form of sediment; no turbidity.

Dextrose: Acid and gas.

Lactose: No acid; no gas.

Saccharose: Acid and gas.

Pathogenicity: No effect on rabbits when injected subcutaneously in doses of 5 c.c., or when given per os.

5. The data in regard to the effect of cultures on horses has already been published by Dr. John S. Buckley of the Bureau of Animal Industry, in the Journal of American Veterinary Medical Association, March, 1917, New Series, Vol. 3, No. 7. His paper was first presented at the meeting of the A. V. M. A., Detroit, Mich., Aug. 21-25, 1916. For reasons apparent in the conclusions, this data is now reprinted. This part of the work was performed in order to demonstrate whether or not the organism may be the causative factor in the disease of horses commonly known as forage poisoning.

This yeast was grown on wort agar, and whenever transfers were made to preparations containing the Nevin organism a loop was used, since it had been previously determined that the latter organism grew irregularly when small quantities of the yeast were collected with a wire. The culture employed was that recovered from the heart's blood of the first rabbit, and which had been used in the case of the horse.

The cultures were kept under aerobic conditions on top of the incubator covered by a black cloth to protect them from the light. The temperature ranged from 25 to 32 C.

The mediums on which the mixed cultures of the Nevin organism and the yeast were grown consisted of ordinary 1 per cent. lactose agar slants and the L. R. W. broth already described. A trace of dextrose was probably present in both instances.

Transfers were first made from an anaerobic lactose agar preparation of the Nevin organism to two tubes of the L. R. W. broth. To one tube was added a heavy inoculation from a wort agar culture of the yeast.

After three days turbidity appeared in the preparation containing both bacillus and yeast, and a hanging drop showed the presence of motile bacilli. The yeast was present in the sediment, but only in very small numbers in the supernatant fluid. In the tube inoculated with the anaerobe alone no turbidity appeared during ten days, and no trace of the organism could be observed microscopically, indicating that no aerobic contamination existed in the anaerobic culture from which the aerobic preparation was made.

The mixed culture was now transferred to two lactose agar slants, and to one of them was added a loopful of the yeast.

After forty-eight hours a few colonies of the yeast were observed on the slant to which no additional yeast had been conveyed. Microscopically, yeast cells, but no bacilli, were seen. The other tube to which had been carried a heavy transfer of yeast showed an irregular growth in which appeared microscopically a few motile bacilli, without spores, mingled with the yeast cells.

This preparation was now transferred to two slants of the same medium over one of which, as in the previous case, a loopful of the yeast was smeared. After forty-eight hours the simple transfer showed microscopically many yeast cells and a few nonmotile bacilli without spores. The other tube to which the yeast had been conveyed showed the same picture except that motility was observed in the bacilli.

The latter preparation was transferred to a fresh tube of lactose agar with the addition of yeast as in the previous cases. No microscopic observations were made. After three days this culture was again conveyed to two slants of lactose agar, and to one of these yeast was added. At the end of forty-eight hours both these tubes showed irregular, heaped-up growths. Microscopically, there were seen numerous bacilli along with a few spores in addition to the yeast cells.

This process was repeated, and in forty-eight hours both the simple transfer and the culture with additional yeast showed microscopically equally numerous motile bacilli mixed with the yeast. Therefore, the further addition of yeast was not considered necessary, and the simple transfer was selected and conveyed by means of a loop to a fresh lactose agar slant.

In this preparation after three days the motile bacilli were found to be present in small numbers. From this tube another transfer was made, and again in three days the same picture was seen, except that some spores were noted as well.

This culture was transferred to a final lactose agar slant and then the medium was changed in order that the organism might be given an opportunity to produce its toxin in a liquid. After two days' cultivation the final lactose

agar preparation was carried with a loop along with a fresh supply of yeast to L. R. W. broth. Then after four days, and again after three days, transfers were made, with addition of yeast, from L. R. W. broth culture to L. R. W. broth.

In this latter preparation turbidity and sedimentation appeared in forty-eight hours. On the third day this increased, and microscopically in the supernatant fluid were seen motile bacilli with subterminal spores. In the sediment were yeast cells.

Thirty-two days had now elapsed since the first aerobic transfer and during this period the bacillus had been successfully cultivated for seventeen days on lactose agar slants by means of eight transfers.

The toxicity of the last broth culture of the Nevin organism and the yeast was next tested:

One c.c. of this culture was given by stomach tube to a rabbit weighing 1,760 gm.; 1 c.c. was injected into a piece of meat and given to a cat to eat.

No symptoms were noticed in the cat during a period of a week after ingestion of the food, but the rabbit began to show slight sluggishness after three days, and on the succeeding day was found dead in his cage.

The postmortem showed the usual findings somewhat intensified and there was also observed a marked congestion of both lungs, with the exception of part of one lobe, and a hemorrhagic pericarditis with blood clots. The surface of the kidney presented a curious pitted appearance. Smears and anaerobic and aerobic cultures from the heart's blood, liver and spleen were negative.

In the meantime the aerobic mixed culture was carried on in L. R. W. broth as formerly. That is to say, a fresh transfer with the addition of the yeast was made every three or four days. After three such transfers, at the end of a further period of twelve days, 1 c.c. of a three-days' culture was given by stomach tube to a rabbit weighing 2,180 gm. The next morning the animal was found dead. The postmortem was performed on the following day and showed a marked hemorrhagic congestion of the lungs and bloody fluid in the pericardium, with injection of the blood vessels of the heart wall and duodenum. Postmortem decomposition had set in and masked the findings.

At the end of another forty-eight hours 1 c.c. of the next transfer was dropped on a little oatmeal and given to a rabbit weighing 2,265 gm. The oatmeal completely absorbed the fluid and was entirely consumed by the following morning. Symptoms of weakness developed on the fourth day and the animal ceased to eat, although his jaws were observed to move as if chewing the food. After another period of two days this attempt also ceased, and during the next week the food placed in the rabbit's cage remained untouched. No symptoms other than those of weakness were observed. Then improvement set in and the animal was discarded after another week had elapsed. At this time he was able to eat and seemed apparently normal.

The next transfer was used to test the toxicity of the preparation for donkeys: 0.2 c.c. of the culture when three days old was placed in about 10 c.c. of sterile saline solution and given to a donkey by pouring on a little bran. The bran was consumed within two hours after being treated with the culture. As a control, 1 c.c. was placed on some uncooked oatmeal and given to a rabbit weighing 1,520 gm.

The rabbit developed no symptoms till the end of the second day, when a retraction of the neck was noted. On the following morning he was found dead. Postmortem, the animal showed the usual general injection of the blood vessels, and as in the first two rabbits, the lungs were congested. Hemorrhage had occurred in the pericardium. The kidney and liver were congested and the spleen was enlarged and of a pulpy consistency. Neither the Nevin organism nor the yeast could be recovered by anaerobic or aerobic methods.

The donkey became sick at the end of four days after the feeding. At this time he was seen by the caretaker of the stable to be lying down. The writer was not able to see the animal until another forty hours had elapsed, six days after ingestion of the culture. At this time the donkey was prostrate, breathing heavily, with dilated nostrils, from which a mucous fluid was dribbling. The animal was too weak to rise or walk unaided. When raised, he took a few steps supported by several men. He was then pushed back in the stall, where, after standing for ten or fifteen minutes, he fell. No symptoms indicating paralysis could be observed. On the following morning the donkey was dead. Opportunity for a postmortem did not occur before the next day, when decomposition completely masked any lesions that might have been present. No cultures were taken. The temperature chart for the first four days follows:

TEMPERATURE, DONKEY 1

Date	A. M.	Noon	P. M.
Feb. 15 '16 p. m.	Culture ingested.		98.2
Feb. 16	97.2	97.0	97.6
Feb. 17	97.2	96.4	98.6
Feb. 18	97.2	96.6	99.0
Feb. 19	96.0	97.8	96.8 marked ill-
Feb. 20			ness
Feb. 21			
Feb. 22	Death.		

In the meantime the mixed culture of *B. botulinus* and the yeast had been carried on in the usual manner by transferring every third day to L. R. W. broth, with the addition of yeast at each transfer. After two such transfers 1.0 c.c. was given on oatmeal to a rabbit weighing 1,460 gm. as a control, and 0.5 c.c. was given on bran to a second donkey.

The rabbit developed a slight weakness in the hind legs on the following day and was found dead the next morning, less than forty-eight hours after eating the food. The postmortem was performed at once, but decomposition had already set in. However, injection of the blood vessels in the subcutaneous region, in the diaphragm and around the wall of the duodenum could be made out. The lungs showed areas of congestion. Aerobic and anaerobic cultures from the heart's blood were negative.

The donkey on the second morning after eating the infected bran, that is to say, after a lapse of about forty-two hours, was observed to paw the earth and tremble in a manner to indicate the possible existence of some intestinal colic. No other symptoms were manifest. On the evening of the same day he seemed sluggish, but presented no definite signs of illness. However, on the morning of the third day, about sixty-six hours after eating the bran, he was found with the tongue completely paralyzed, protruding about 3 inches or more from the mouth. Food and drink were refused. The pulse was about 30 and somewhat weak. The temperature was 99. The sluggishness was more marked than on the preceeding evening, but there were no signs of paralysis other than those evident in the tongue. At 2 p. m. of the same day the animal was noticed by the watchman, standing in his stall, the condition apparently unchanged. At 4 p. m. he was seen by the writer. At this time the donkey was lying down, too weak to rise without aid. When forcibly lifted, he was unable to stand alone, and when put back on the ground lay quietly, with apparent consciousness of his surroundings. The tongue still protruded, but no other signs of paralysis could be noted. About midnight the donkey died, in the neighborhood of three days and eight hours after eating the bran, and fifteen hours after the first serious symptom, paralysis of the tongue, was observed. The temperature chart, taken by the stable attendant, follows:

TEMPERATURE, DONKEY 2

Date	A. M.	Noon	P. M.
Feb. 21 '16 p. m., culture ingested.			99.2
Feb. 22	98.0		99.0
Feb. 23	98.0		98.0
Feb. 24	99.0	97.8	97.0 Death 12 M.

The postmortem was performed by Dr. Buckley about ten hours after death had occurred, before any signs of decomposition had set in. The following points were noted:

Subcutaneous vessels not markedly injected.

Stomach: Vessels in outer wall injected. Inner wall shows areas of shallow erosions. Mucous membrane is quite pale and coated with mucus.

Duodenum: Outer wall shows areas of hemorrhage in patches. On inner wall are punctate and larger areas of hemorrhage.

Jejunum: Outer wall like that of duodenum, except that areas of punctate hemorrhage are present in addition to the larger patches. The inner wall shows a condition intermediate between the duodenum and ileum.

Ileum: Outer wall like that of jejunum, but lesions are more marked. On inner wall is seen a condition of diffuse hemorrhagic enteritis.

Cecum: Outer wall normal. Inner wall shows numerous punctate hemorrhages.

Colon: First part reddened on outside. Stained on inside. Second and third parts normal and full of feces.

Mesentery: Larger vessels are injected.

Spleen normal.

Liver slightly congested.

Kidney markedly congested.

Diaphragm, no marked injection.

Lungs markedly congested.

Heart wall, no points of hemorrhage.

Pericardium normal.

Bladder full but not distended.

Cerebrospinal fluid, normal in quantity at junction of head with body.

Cultures were made from the heart's blood, liver and spleen in L. R. W. broth, on plain agar and on wort agar. The L. R. W. broth cultures were placed under anaerobic conditions. The others were kept aerobically. All were kept at a temperature of between 25 and 30 C. On none did any growth develop.

These experiments indicate that the organism, although in pure culture an obligatory anaerobe, may be cultivated in mixed culture with a yeast under aerobic conditions such as exist on an aerobic slant agar preparation.

Effect of Light on Toxin Production.—The effect of light on the production of toxin was next studied. The medium employed consisted of 2 per cent. lactose R. W. broth of reactions varying from neutral to about +2. Traces of dextrose were present. About 10 or 15 c.c. were kept in each tube. A tube of this medium was inoculated from a dextrose agar preparation of the same strain used in the previous experiments, and with the yeast from a twenty-four-hour wort agar culture. Other tubes were inoculated with simple transfers of the two organisms. All the preparations were kept on the desk in front of a window at temperatures ranging from 20 to 30 C. The window faced the North, and the sunlight did not fall on the cultures.

In the tube containing the Nevin organism alone no growth developed. In the tube to which the yeast had been transferred there occurred a sediment without turbidity. Microscopically only yeast cells could be demonstrated. It may, therefore, be inferred that the cultures used contained no aerobic contamination capable of growing in broth.

In the tube containing both anaerobe and yeast there developed in forty-eight hours the usual turbidity, sediment, and foul odor. The hanging drop revealed the presence of motile bacilli with subterminal spores, and yeast cells.

After the culture was five days old 1 c.c. was dropped on some uncooked oatmeal and given to a rabbit weighing 1,620 gm. This was consumed by the following morning. Twenty-four hours later the rabbit ceased to eat. After another interval of forty-eight hours the animal was found dead. From the condition of the body death probably occurred between forty-eight and seventy-two hours after eating the oatmeal. Postmortem there was observed the decomposition that was to be expected and the usual injection of the blood vessels and engorgement of the liver. The lungs were markedly hemorrhagic, only a few areas being unaffected.

The culture was kept alive by transferring every five or ten days to fresh L. R. W. broth with the addition of yeast. The tubes were kept on the desk in the light under aerobic conditions. After being thus grown for twenty-three days a five-day culture was given in dose of 1 c.c. by stomach tube to a rabbit weighing 1,785 gm. No symptoms developed until the afternoon of the fourth day after the feeding. Then the animal was observed to make continual efforts to swallow without eating. The following morning this persisted and in addition the neck was retracted. There was no paralysis of the limbs. At 3:30 p. m. the rabbit was found dead and stiff, with neck retracted.

The postmortem showed a tumor above the umbilicus connecting with the region of the spine and a hemorrhagic fibrinous exudate beneath the skin. The peritoneum, mesentery, diaphragm and heart wall were injected. The heart wall was congested. The lungs were normal, except for one small area of hemorrhage. The liver was congested. The spleen was normal. Cultures made anaerobically and aerobically from the heart's blood were negative.

After being grown in the light on the desk for two and one-half months, with transfers every five or ten days, 5 c.c. of a fourteen-day culture were given by stomach tube to a rabbit weighing 1,890 gm. No symptoms were observed the following day. At 4 p. m. the rabbit ate part of a carrot. The next morning, less than forty-eight hours after ingestion of the culture, it was found dead, with eyes open, lying on the side, without any signs of a struggle. The carrot given the previous afternoon was half consumed. Postmortem, there was seen the usual injection, and in addition there was one large area of hemorrhage on the upper gut. The lungs were engorged. The heart swollen, firm, congested. The vessels of the diaphragm were markedly injected. The spleen and kidney were about normal.

These experiments indicate that exposure to daylight for two and one-half months does not inhibit the toxin production to any marked degree.

Effect of Heat on the Toxin.—The effect of heat on the toxin and on the toxin-forming power of the bacillus was now taken up.

A five-day aerobic mixed culture in L. R. W. broth grown at temperatures under 26 C. was selected. As a control, 1 c.c. of this culture was given by stomach tube to a rabbit weighing 1,785 gm. Then the preparation was heated to from 65 to 70 C. for twenty minutes, and 2 c.c. of this were injected subcutaneously into a rabbit weighing 2,460 gm. The control developed symptoms four days later and died on the following day. The postmortem showed the usual findings. The other rabbit failed to develop any symptoms during eight days following the inoculation.

This experiment was repeated, with the variation that the heated culture was given per os instead of subcutaneously, and the period of observation lengthened in order to determine the ability of the spores to develop in the rabbit's intestines. Ten c.c. of a thirteen-day mixed culture grown aerobically in L. R. W. broth at a temperature of 30 C. in the incubator and heated to from 65 to 70 C. for twenty minutes were given by stomach tube to a rabbit weighing 2,400 gm. At the end of six weeks no symptoms had developed and the rabbit weighed 2,335 gm.

These experiments confirmed those of many other workers in regard to the destruction of the toxin by moderate amounts of heat, and indicated that the spores of the organism were not pathogenic when given subcutaneously or per os.

Regaining Toxicity After Heating.—To demonstrate the possibility of a heated culture regaining its toxicity, the following experiments were undertaken.

A five-day mixed culture grown aerobically on the desk at temperatures below 25 C. was heated to from 65 to 70 C. for twenty minutes. As a control, 2 c.c. of this preparation were placed on raw oatmeal and given to a rabbit weighing 1,980 gm. The food was not entirely eaten till three days had elapsed, but since no symptoms developed in the ensuing four days, and since other experiments had shown that heated cultures were not toxic, it may be concluded that in this case as well the heat applied had rendered the toxin harmless.

Since in the original heated culture the yeast had been killed, the remaining spores of the anaerobe could not be expected to develop aerobically and produce toxin without the addition of a fresh supply of yeast. Accordingly, immediately after cooling, a fresh transfer of yeast was made to the culture and the preparation placed on the desk. After a week had elapsed, during which time the temperature ranged under 26 C., 1 c.c. of this preparation was placed on oatmeal and given to the same rabbit, whose weight was now 1,970 gm. The food was consumed during the next forty-eight hours, but no marked symptoms developed during the six days following the giving of the culture. To determine its toxicity finally, 2 c.c. of this same preparation, thirteen days having passed since the heating, were injected subcutaneously into the rabbit at 3 p. m. The following morning the animal was found dead, lying stretched out in the cage with no evidence of convulsions having occurred. Death had ensued in from twelve to eighteen hours.

Postmortem there was observed a serofibrinous exudate beneath the skin, without noticeable hemorrhage. There was some injection of the peritoneum and mesentery. The heart was swollen, but not otherwise abnormal. The lungs showed no congestion. The diaphragm was thickened, and the vessels injected. The liver was congested, the spleen markedly enlarged and congested, the kidneys congested, the bladder full and the vessels injected. Cultures from the heart's blood were negative.

On account of the failure of the control rabbit to eat the oatmeal immediately on being offered, and on account of too short a time being allowed for observation, it is not possible to draw any very definite conclusions from this preliminary experiment. However, it would indicate that contaminated food heated sufficiently to destroy the toxin but not to kill the spores may again become toxic.

Effect of Drying on Toxicity.—To test the effect of drying on the toxin-producing power of the bacillus a small piece of filter paper was saturated with a seven-day mixed culture made in L. R. W. broth. This was placed in a sterile test tube and allowed to dry out on the desk for twenty-two days. Then it was transferred to a tube of L. R. W. broth along with additional yeast. This preparation was placed in the incubator at 30 C. After four days, turbidity and odor were noted.

When the culture was seven days old 2 c.c. were given by stomach tube to a rabbit weighing 1,505 gm. No symptoms appeared the following day. On the second day no observations could be made. On the third day, seventy-two hours after ingestion, the animal was found dead. No postmortem could be performed. Apparently drying for twenty-two days fails to destroy the toxin-producing power of the organism.

Toxin Production at Different Temperatures and Reactions.—According to Dickson, *B. botulinus* may produce its toxin in medium of reaction of over +3,

and according to Nevin the organism isolated by her produces toxin at a temperature of 37 C. To test these observations broth was made according to the following formula:

	Gm. or C.c.
"Liebig's" extract	5
Armour's peptone	10
Salt	5
Water	1,000

Different lots were titrated to 0, +1, +2, +3 and +4 cold, and 10 c.c. measured into tubes. After autoclaving, a tube of each titration was inoculated from a mixed culture of the Nevin organism and yeast grown four days in L. R. W. broth at 30 C. and an additional transfer of yeast was added. The preparations were placed in the incubator at 38 C. In twenty-four hours faint turbidity without odor appeared in the tube of reaction 0. More marked turbidity with odor appeared in tubes of reaction +1 and +2, and no turbidity in tubes of reaction +3 and +4. Since the yeast grows poorly in neutral or alkaline medium and since in pure culture the Nevin organism will not grow aerobically, it was to be expected that a moderate acidity would give the best symbiotic growth.

In seventy-two hours turbidity and some odor was observed in the +3 tube. On examination in the hanging drop, tubes of reactions 0, +1 and +2 showed large motile bacilli, extracellular spores and yeast cells. Tube +3 showed fewer, larger bacilli with intracellular spores and yeast cells, and tube +4 only yeast cells without bacilli.

When the cultures were seven days old a transfer was made from the +3 preparation to a similar tube of +3 broth and additional yeast added. This was likewise placed in the incubator at 38 C. In it there developed turbidity and odor, and when five days old 1 c.c. was taken and given by stomach tube to a rabbit of 1,730 gm. weight. At the same time a further transfer to +3 broth tube was made with addition of yeast. During the next six days no positive symptoms developed in the rabbit, so at the end of this period 5 c.c. of the six-day old culture, made when the first dose was given the rabbit and grown at 38 C., were given to the same rabbit by means of a stomach tube. The following day the animal was sluggish, and in forty-eight hours it ceased to eat. In seventy-two hours, on the morning of the third day after ingestion of the last dose, it appeared very apathetic. The muscles were relaxed and it did not eat. No paralysis was observed. At 3 p. m. the same day it was very weak, the muscles of the abdomen greatly relaxed, and the breathing labored. There was no paralysis. On being placed on the floor it hopped quite energetically, but on being returned to the cage died in convulsions as if from embolus.

Postmortem, the injection in the mesentery around the gut was very marked. The liver and spleen were engorged. The diaphragm was injected. The lungs appeared normal except for one small dark area in one lobe that resembled an infarct. The heart was distended and engorged. Transfers from the heart's blood were negative.

This experiment confirms the observations of Dickson and Nevin in regard to reaction and temperature. It would indicate that in the absence of sugar a weakened toxin is formed in meat extract broth of reaction of +3 at 38 C.

These points do not coincide with the observations of van Ermengem and others in regard to the original strain of *B. botulinus* and indicate that while the organism isolated by Nevin may be a variety of *B. botulinus*, its requirements for toxin production make it theoretically a much more dangerous organism. For since it can produce

toxin at body temperature under aerobic conditions in a medium of +3, it is possible that it might grow in the human intestine, or in the throat in symbiosis with an appropriate aerobe.

That it fails to produce toxin in the intestines of the rabbit has been shown in an experiment already recorded. This may be due to some inhibiting effect of intestinal organisms such as *B. coli* or *Bact. welchii*. In the throat its growth would to a great extent depend on its power to multiply in symbiosis with streptococci, *Micrococcus aureus* and other bacteria commonly found in infections, and to its ability to withstand the inhibiting influences which the body tissues possess.

Opportunity to solve all these questions was not available. To test the ability of the Nevin organism to produce toxin in the presence of *B. coli*, *Bact. welchii*, and *M. aureus*, the following experiments were undertaken.

Toxicity in Presence of Other Organisms.—In the first series sterile milk was used as a medium. About 10 c.c. were placed in each tube. One was inoculated from an eight-day broth culture of *B. coli* and with a mixed culture of the Nevin organism and yeast grown eight days aerobically at 30 C. in L. R. W. broth. To another tube was transferred the Nevin organism and yeast culture, along with a transfer from a three-day wort agar culture of the yeast. Two other tubes were inoculated with single transfers from the *B. coli* and the yeast culture as controls. All were kept aerobically at 30 C.

In the course of a week the yeast caused no change in the milk. In twenty-four hours *B. coli* produced acidity with slight reduction. In seventy-two hours no coagulation occurred. In thirteen days there was coagulation, with little whey formation, together with some reduction of the litmus and a sweetish odor.

In twenty-four hours the yeast and Nevin organism caused no change. In seventy-two hours there was about 90 per cent. peptonization. After thirteen days there appeared a heavy scum. The odor was very foul.

The yeast, Nevin organism and *B. coli* culture caused a reduction in the litmus in twenty-four hours. In seventy-two hours it produced coagulation with little whey formation. In thirteen days the milk was 50 per cent. peptonized. The odor was sweetish. Here the peptonization would indicate the growth of the Nevin organism, but the lack of foul odor would indicate the opposite.

Hanging drops made from the whey of the yeast and Nevin organism preparation showed bacilli of the general type of *B. botulinus* with subterminal spores. There was no motility. The yeast was present. No explanation of the scum was found unless it was formed by the yeast.

Hanging drops from the whey of the *B. coli*, Nevin organism and yeast culture showed many large and small nonmotile bacteria and many yeast cells. There were no spores and no bacilli of the general morphology of *B. botulinus*. Apparently *B. coli* had inhibited the growth of the Nevin organism. As a final test the two cultures, now evaporated down to about 7 c.c., were given to two small rabbits by stomach tube. The yeast and Nevin organism was given to a rabbit weighing 910 gm. The *B. coli*, Nevin organism and yeast preparation was given to one of 955 gm. weight. The cultures at the time were fourteen days old.

The following morning, twenty-four hours after ingestion, no symptoms were observed in either rabbit. At midday both were well. At 2 p. m. of the same day the rabbit treated with the yeast and Nevin organism preparation was

found dead. The postmortem revealed slight subcutaneous injection. Around the gut this was more marked; the liver was engorged; the diaphragm injected; the upper lobes of the lung showed a hemorrhagic condition; the heart was still beating when examined; it was apparently normal. Cultures made from the heart blood were sterile.

At 4:30 p. m. of the same day the remaining rabbit was lively. At 9 a. m. of the following morning it was found dead and stiff, and had apparently been dead some time. Less than forty-eight hours had elapsed since the ingestion of the culture. Postmortem decomposition had set in. The animal was very rigid. Injection of subcutaneous and mesenteric vessels was present; the liver was engorged; the spleen normal; the heart normal; the upper lobes of the lungs were markedly hemorrhagic.

It is therefore evident, in spite of the Nevin organism not being demonstrable microscopically in the mixed culture, that *B. coli* has not the power of suppressing the growth of the Nevin organism in milk.

The experiment in regard to *Bact. welchii* was not completed by animal testing on account of lack of time. In the preliminary work *Bact. welchii* was first grown aerobically with the yeast in broth, and this was used as a stock culture. Tubes of plain broth of reaction +1, of neutral L. R. W. broth, and of 2 per cent. dextrose broth were inoculated from a twenty-four-hour culture of *Bact. welchii* and yeast, and from an eight-day culture of the Nevin organism and yeast in neutral L. R. W. broth. One set was placed at 30 C., one at 39 C. After twenty-four hours, motile bacilli could be demonstrated in the neutral, L. R. W. and plain broth cultures kept at 30 and 39 C. But in the 2 per cent. dextrose cultures, both kept at 30 and 39 C., no motile bacilli of the morphology of *B. botulinus* could be demonstrated, but only nonmotile fat bacteria of the type of *Bact. welchii*. This would indicate that *Bact. welchii* cannot inhibit the Nevin organism in the presence of lactose alone, or in the absence of sugar, but that it has some inhibitory power in the presence of dextrose. However, the lack of animal tests renders this inconclusive. Yet it is possible that the presence of both dextrose and *Bact. welchii* in the rabbit's intestine is a cause for the nonpathogenicity of the Nevin organism under these conditions.

In regard to the growth of the Nevin organism in symbiosis with *M. aureus*, the following experiment was performed.

A tube of plain extract broth of acidity +1 was inoculated from pure agar cultures of *M. aureus* and the Nevin organism and placed at 38-39 C. The *M. aureus* strain selected was one which had produced typical lesions in the rabbit. In twenty-four hours turbidity appeared and in the hanging drop could be seen motile bacilli with subterminal spores. In seven days the hanging drop showed cocci, extracellular spores and nonmotile bacilli of the general morphology of the Nevin organism. Five c.c. of this culture were given by stomach tube to a rabbit weighing 2,419 gm. On the following day no symptoms developed. Two days later, three days after ingestion of the culture, the rabbit was found stiff and decomposed. It had evidently died between twenty-four and forty-eight hours after ingestion. In spite of the decomposition there could be made out marked injection of the vessels in the subcutaneous tissue, mesentery and diaphragm; the liver was engorged; the spleen was somewhat enlarged; the kidneys pitted; the upper lobes of the lungs were markedly engorged; the lower lobes were not affected. No cultures were taken.

SUMMARY

The organism isolated by Nevin from cottage cheese that had caused an epidemic diagnosed postmortem as botulism is a distinct variety of the *B. botulinus* of van Ermengem.

It differs from this organism in the following particulars:

It is more toxic for rabbits.

It is less toxic for cats.

It grows and produces toxin at 37 C. and in sugar-free media of acidity of +3.

In the rabbit it produces symptoms and lesions not identical with those described by van Ermengem.

Although stock cultures obtained as *B. botulinus* were found non-toxic, the organism of Nevin produced toxin after resisting the effect of light for two and one-half months and of drying on paper for twenty-two days.

In symbiosis with a yeast, it can be cultivated aerobically on agar slants. Grown with *B. coli* and a yeast in milk, it produces death when ingested by a rabbit. Grown with *M. aureus* in extract broth at 38-39 C., under aerobic conditions it likewise produced death when ingested by a rabbit.

In numerous instances the toxin produced death in the rabbit without any preliminary symptoms being observed. In one case in which the toxin was produced in +3 broth, death apparently was associated with infarct of the lung and embolus.

The nonpathogenicity of this strain is not definitely proved. In the intestines of the rabbit the organism apparently fails to grow, and injected subcutaneously the spores produce no symptoms. Yet the fact that it grows at body temperature in the presence of *M. aureus* renders it possible that in mixed infection in the throat or protected from the influence of the body tissues in some cavity of the teeth it may produce its toxin.

In the writer's experience few symptoms are to be observed in the rabbit when the toxin given is formed under aerobic conditions. Under these circumstances no definite paralysis of the limbs was observed, although the organism produced this symptom when grown anaerobically.

The postmortem findings of Ophüls and Dickson indicate that certain strains of *B. botulinus* may produce thrombosis and hemorrhage in the meninges and central nervous system.

These facts would seem to indicate that the toxin produced by the Nevin Strain might cause sudden death by embolus or hemorrhage in the brain, accompanied by but few preliminary symptoms of a minor character.

The culture is capable of killing donkeys when given on feed in doses as low as 0.2 c.c.

What is known of the etiology, symptomatology, and pathology of forage poisoning indicates that the disease may be caused by a toxin similar to that produced by *B. botulinus*.

No definite conclusions can be drawn from the few experiments performed, but in view of the facts mentioned it seems probable that some cases of forage poisoning may be due to certain strains of *Bacillus botulinus*.⁶

Finally, it should be observed that this organism presents manifold possibilities for use as a poison both for horses and men. The toxin of the variety of *B. botulinus* employed certainly possesses the power of killing horses in very minute doses when given in the feed.

In regard to the toxicity of the organism for man, it would seem at first glance that the symptoms produced would be those of botulism. For the organism was isolated by Nevin from cheese that had produced numerous cases of a disease diagnosed postmortem as botulism, and the symptoms of this disease are well known and should be capable of diagnosis.

But there always remains the possibility of the organism growing in the body and producing its toxin slowly and under altered conditions, thereby causing a different syndrome from that seen when the toxin is ingested.

It is noteworthy that the most marked, constant lesion produced in the rabbit, horse and donkey was a congestion of the lungs, *when the culture was ingested by the animal*. In the first preliminary experiment on the rabbit, and in one other in which the culture was injected subcutaneously, this lesion was not noticed, nor is it apparent in Nevin's⁴ work in which injections were used throughout. Apparently then, the organism is capable of producing various lesions. Certainly the pathologic findings in rabbits which had ingested cultures grown in symbiosis with a yeast under aerobic conditions would not lead one to make the diagnosis of botulism if these lesions had been found in man. Again, the dosage would probably affect the symptoms. In large doses sudden death might be expected.

If placed in the water supply the organism might find conditions favorable for the production of a small amount of toxin, and since disinfectants have little effect on spore bearers, it is probable that treatment of such water by the hypochlorites would fail to kill the organism.

6. Since this statement was written the work of L. H. Pammel (Forage Poisoning Due to *Bacillus Botulinus*, Am. J. Vet. M. **13**: No. 3, March, 1918) has strengthened the supposition.