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STUDIES ON ORGANISMS CONCERNED AS CAUSATIVE FACTORS IN BOTULISM

BY

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STUDIES ON ORGANISMS CONCERNED AS CAUSATIVE FACTORS IN BOTULISM.

By IDA A. BENGTSON, Associate Bacteriologist, Hygienic Laboratory, United States Public Health Service.[1]

I. CULTURAL AND IMMUNOLOGICAL STUDY OF STRAINS OF ORGANISMS CONCERNED IN BOTULISM.

INTRODUCTION.

The discrepancies in the literature regarding the identification of the organisms which have been concerned in the production of the disease known as botulism and the isolation of a new "type" by the writer, which in many of its characteristics varied from the usual American strains designated as *Clostridium botulinum*[2] types A and B, prompted a more complete investigation of the so-called "botulinus" strains than has hitherto been attempted. The strains studied include representatives of several different groups, the purpose being to carry out as thorough a study as possible with strains of undoubted purity rather than to work with a large number of strains. The questionable purity of American strains rendered the use of single cell cultures necessary. It is possible that other types of this group of organisms may be found later, and if such is the case the work here reported may serve as a basis for identifying and classifying such organisms. The cultures studied include representatives of four different combinations of characteristics, there being two groups on the basis of cultural behavior and three on the basis of the kind of toxin produced.

Representatives of each of the groups will first be considered in a general way, after which a comparative cultural and immunological study of a number of strains of each group will be taken up.

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[1] Manuscript submitted for publication Aug. 6, 1923.

[2] The use of the generic name *Clostridium* has been adopted in preference to *Bacillus* in accordance with the recommendation of the Committee on classification of the Society of American Bacteriologists. The subdivision of the spore-bearing rods into *Bacillus* for aerobic forms and *Clostridium* for anaerobes or microaerophiles is a logical one. Subdivision on this basis is more justifiable than on the basis of spore location, which is sometimes variable in the same species. For the same reason the use of the two generic names *Clostridium* and *Plectridium* for anaerobes (the former for those whose spores cause the cell to become spindle-shaped and the latter for those in which the spore causes an enlargement of one tip of the cell), as recommended by Buchanan, does not seem advisable. *Clostridium*, as applied to certain of the strains of organisms concerned in botulism, is particularly appropriate on morphological grounds in accordance with Zopf's definition of *Clostridium* (spindle-shaped, ellipsoidal, or tadpole-shaped). The use of the generic name *Ermengemillus* by Heller as applied to the proteolytic American A and B types is probably not justifiable for the reason that the organism described by van Ermengem is not identical with the American A and B types, as will be brought out in the cultural study.

DESCRIPTION OF VAN ERMENGEM STRAIN.

The organism originally described by van Ermengem and named by him *Bacillus botulinus* should be considered as one of the type species of this group. Cultures of this organism were obtained by van Ermengem in an outbreak of botulism occurring in Ellezelles (Hainaut), Belgium, in 1895, which involved 34 cases, with 3 deaths. The organism was isolated from the ham which was the cause of the outbreak and from the spleen and contents of the stomach and large intestine of one of the fatal cases. In van Ermengem's articles the following description of the organism is given: A very large bacillus 4–6 by 0.9–1.2μ, with rounded extremities. It sometimes occurred in pairs and rarely in filaments. The spores were generally terminal and somewhat wider than the rod and resistant to the action of ordinary stains. Clostridial and spindle forms were sometimes observed. Motility was slight, and there were four to eight very fine flagella. The organism was gram-positive, but the decolorizing action of alcohol was very rapid. Characteristic colonies were formed on glucose gelatin, which were round, transparent, yellowish brown, and contained granular bodies which were in continual motion. Gelatin was liquified, but milk was not changed nor coagulated. Gas was produced in glucose agar. In carbohydrate broths containing lactose or saccharose no gas was formed, but abundant gas formation was present in glucose broth. The organism was a strict anaerobe. An odor of butyric acid was produced in various media. In van Ermengem's earliest publication (1896) this was described as slightly rancid and not unpleasant.

A reaction of the medium acid to litmus or phenolphthalein prevented growth, while a medium containing about 1.43 per cent of Na_2CO_3 afforded a good growth with toxin production. The optimum temperature was between 20° and 30° C. Temperatures of 37° to 38.5° C. were unfavorable for normal development. There was no spore formation, and involution forms were present and scarcely any toxin was produced at this temperature. The spores were destroyed by a temperature of 85° C. maintained for one-fourth hour or 80° C. maintained for one-half hour.

The toxin when fed was very toxic for guinea pigs and mice. Larger doses were required to produce symptoms in rabbits. Rats and pigeons could withstand very large doses, as could dogs, hens, and cats. Frogs and fish were absolutely refractory. Rabbits, guinea pigs, mice, pigeons, and cats showed typical symptoms on subcutaneous injection. Dogs, rats, and chickens were resistant to very large doses. Monkeys (Rhesus) were susceptible both to subcutaneous inoculation and to feeding.

Several other strains were isolated in later outbreaks of botulism occurring in Europe, which differed in certain cultural respects from the strain originally described by van Ermengem. This was true particularly in regard to the proteolytic activity of the organism. Von Hibler studied three strains which were found to coagulate and digest the casein of milk. The fact that one of the strains studied was stated to be the van Ermengem strain has tended to obscure the real facts in regard to the nature of the culture isolated by van Ermengem. According to van Ermengem's own description the culture which he studied was nonproteolytic toward casein, and it is probable that a contaminant entered later. The other two cultures studied by von Hibler may or may not originally have been proteolytic.

One of the cultures studied by von Hibler was the Darmstadt strain, which represented another "type" as regards toxin produc-

tion. The toxin produced by the van Ermengem strain was not neutralized by the antitoxin of the Darmstadt strain and vice versa.

The Darmstadt organism was concerned in an outbreak occurring in Darmstadt, Germany, in 1904, in which 21 persons who consumed a bean salad developed symptoms of botulism, among whom 11 died. The organism was described by Gaffky and Landmann. Landmann considered the organism very closely related to van Ermengem's, though he states that there were occasionally small differences in growth. Cultures incubated anaerobically were described as having an odor of Limburger cheese. Other authors have also noted some differences. Leuchs (1910) found the Darmstadt strain less gram-positive than the van Ermengem strain. It also grew readily and produced toxin at 37° C. Ornstein, comparing the two cultures, also noted the fact that the Darmstadt strain did not retain the gram stain as well, that the rods were more slender, that the flagella were different, and that it failed to produce gas in glucose agar. It was also more fastidious in its growth requirements.

Van Ermengem in his final publication (1912) on *Bacillus botulinus* admits that there are certain cultural and biological differences between these two strains, and these differences indicate that within the species a certain variability exists, which he considers, however, not to be greater than that existing in many other species of bacteria. He believes that the strains described up to that time should be considered as belonging to one species. In view of the discrepancies noted the description of the organism as originally given by van Ermengem should be considered the authentic description of the true *C. botulinum.*

Neither the original van Ermengem nor the Darmstadt strains is now available, and thus we can not say with certainty that they are exactly like any of the strains now in existence. We also can not make any definite statement as to the "types" of toxin which they produced, and although it is true that two toxin types were known in Europe and two have been known in this country until recently, it does not necessarily follow that the "types" in Europe correspond to those in this country.

Since the original strain is not available for study it becomes necessary to use for a type species the strain which conforms most nearly to the description of the culture first studied by van Ermengem. A study of the literature of the subject and the laboratory investigation here reported indicates that the strain described in the publications of the Medical Research Committee of Great Britain (1917, 1919), which has also been available for this study, most nearly represents the original van Ermengem culture. This culture is the Lister culture No. 94. A slight difference in regard to the degree of motility may be noted. Van Ermengem states that his culture was

very slightly motile. The Lister Institute culture is described as actively motile. Added information is given as to appearance of colony and fermentation reactions which will be referred to later. This culture is a nonproteolytic culture which does not precipitate or digest the casein of milk and which fails to liquefy coagulated egg albumen and certain other proteins.

CHARACTERISTICS OF AMERICAN STRAINS.

The next group of organisms to be considered consists of those which have been isolated in this country. Among these are the Nevin strain isolated in 1914 from cottage cheese and a number of strains isolated by Graham and associates (1917) in connection with studies on forage poisoning. These belong to the type designated as B in accordance with the classification suggested by Burke (1919, *a*). Other strains isolated in this country mostly from human outbreaks of botulism due to the eating of canned food belong to another type designated as type A. These two types are considered together, since they are so similar culturally; in fact, among the cultures included in this study the differences are so slight that it would not be possible to distinguish with certainty one type from the other by this means. The strain described by Kahn, which is a single cell type A strain and which has been studied most in detail, may be taken as a representative of the American type A and B strains. The most conspicuous characteristic of this strain is the fact that it is actively proteolytic, digesting meat, coagulated egg albumen, milk, and serum with the production of strong odors. Morphologically the organism is described as a large, thick, gram-positive bacillus with rounded ends, the spores usually situated subterminally, but a few medially. Spore formation is accompanied by a swelling of the whole organism.

The work of Kahn has established with certainty the fact that pure cultures of American strains of *C. botulinum* may be proteolytic. It is possible that certain American cultures which have been studied have been contaminated with other spore-bearing organisms, such as *C. sporogenes* (Reddish), but all the American type A and B strains described in the literature have been proteolytic and probably many of these strains have been in pure culture.

Another organism producing symptoms in animals which are indistinguishable from those produced by the type A and B organisms has recently been isolated by the writer (1922, *a*).

This organism was isolated in connection with a study of certain material received from Dr. E. W. Saunders, which included fly larvæ of the species *Lucilia cæsar*. This species of fly was used by Saunders, Wisdom, and White in experimental work on limberneck of chickens and was believed by them to be concerned in the transmission of human poliomyelitis.

The organism was found to differ markedly in its cultural characteristics from the American strains previously studied, particularly in regard to its nonproteolytic action. It also grew much less luxuriantly. It resembled van Ermengem's organism except that it grew without producing gas in glucose agar. It also differed in that it was toxic for rats, while van Ermengem's culture was not toxic for this species.

The following tests made with the toxin showed that it was of a type differing from the strains previously described in this country, guinea pigs being used as the test animal:

Amount of filtrate.	Antitoxin.	Potency.	Symptoms.	Death.
0.0001 c. c	1 c. c. polyvalent	Over 2,500 units, type A; 75 units, type B.	6–7 days	12 days.
0.0001 c. c	1 c. c. type B	140 units	do	10 days.
0.0001 c. c	No antitoxin		do	16 days.
0.001 c. c	1 c. c. polyvalent	Over 2,500 units, type A; 75 units, type B.	43 hours	44 hours.
0.001 c. c	1 c. c. type B	140 units	do	45 hours.
0.001 c. c	No antitoxin		27 hours	42 hours.

The isolation of this organism established definitely the fact that symptoms resembling botulism could be produced in animals by a toxin elaborated by a nonproteolytic organism. This again brought up the question as to the nature of the strain originally isolated by van Ermengem and the other European strains; and also the question of the purity of American cultures.

It was then decided to carry out a comparative study of a number of strains using the Barber single cell technique for obtaining with certainty pure cultures of the various strains.

SOURCE OF CULTURES.

The strains studied include representatives of each of several groups[3] as follows:

GROUP 1, TYPE A.

H. L. No.

No. 1. Memphis strain isolated by the writer from olives concerned in the Memphis, Tenn., outbreak.

No. 3. Strain No. 79. Received from Dr. K. F. Meyer, with the following description: "Received as *B. botulinus* type ? No. 95, Lister Institute, Feb. 1921. Obtained by them from Prof. Madsen, originally Institut f. Infect. Diseases, Berlin." The same strain was received later directly from the Lister Institute.

No. 17. Strain No. 619. Received from the Microbiological Laboratory of the Bureau of Chemistry, United States Department of Agriculture. Isolated by Thom, Edmonson, and Giltner from home canned asparagus causing out break of botulism in Boise City, Idaho.

No. 27. Canton strain. Received from the Microbiological laboratory of the Bureau of Chemistry, United States Department of Agriculture; received by them from Doctor Freeman. The organism was concerned in the Canton, Ohio outbreak, due to the eating of contaminated olives.

[3] The use of the term "group" and the numbers used for the groups are simply for the sake of convenience. in referring to the cultures and no significance is to be attached to it.

GROUP 2, TYPE B (AMERICAN STRAINS).

H. L. No.

No. 5. Nevin strain. Received through the Microbiological Laboratory, Bureau of Chemistry, United States Department of Agriculture. This strain is the strain originally isolated by Miss Nevin from cottage cheese.

No. 19. Strain 465. Received from Dr. Robert F. Graham, Department of Animal Pathology, University of Illinois. Isolated from oats.

GROUP 3, (EUROPEAN STRAIN).

No. 21. Lister Institute strain 94. Received through Dr. G. F. Reddish, Virginia Medical College with the following information. "*B. botulinus* No. 94, strain A, Institute of Infectious Diseases at Berlin; received from Miss Robertson, Lister Institute." The same culture was received later directly from the Lister Institute.

GROUP 4.

No. 7. Strain Saunders. Isolated from fly larvæ (*Lucilia caesar* as identified by Doctor Saunders, St. Louis, Mo.). These larvæ were used in experimental work for the production of "limberneck" in chickens, and also for tests on other animals.

No. 9. Strain Saunders. Nontoxic strain isolated by single cell method from preceding strain.

No. 11. Strain 117. Isolated from fly larvæ (*Lucilia sericata*, as identified by Dr. J. M. Aldrich, United States National Museum) obtained from the crop of a chicken which died from "limberneck" in the vicinity of Glen Echo, Md.

No. 13. Strain 121. Isolated from fly larvæ (*Lucilia sericata* as identified at United States National Museum) obtained through Doctor Saunders from Dr. E. W. Wisdom, Oklahoma, from "limberneck" material.

No. 15. Strain 3421. Isolated by Dr. Robert Graham, Department of Animal Pathology, University of Illinois, from the crop of a "limberneck" chicken.

No. 23. Strain 526. Isolated by Dr. Robert Graham, Department of Animal Pathology, University of Illinois, from the stomach contents of a horse which died with symptoms resembling those of botulism.

No. 25. Strain 487. Isolated by Dr. Robert Graham, Department of Animal Pathology, University of Illinois, from the liver of a chicken suffering from symptoms of leg weakness.

PURIFICATION OF CULTURES.

Single-cell cultures were obtained from all of these by the use of the Barber technic. Two or more single-cell isolations were obtained from each of the cultures and these single-cell cultures were tested for toxicity and specificity as to neutralization by antitoxin before carrying out cultural tests. The writer does not feel that entirely satisfactory results could have been obtained in this study without the use of this exact method of obtaining pure cultures. The superiority of this method over the method of single-colony isolation is illustrated by results obtained in the testing the specificity of certain antitoxins involving the study of the cultures used to produce these antitoxins. A certain antitoxin was received to be tested for its potency in respect to type A antitoxin. Some doubt was expressed by the sender as to the specificity of the antitoxin and accordingly

tests were made against both type A and type B toxins. It was found that the antitoxin contained approximately the same number of type A and type B units. The culture labeled type A used for producing the antitoxin was subsequently obtained and both type A and type B single-cell isolations were obtained, though the sender had not been successful in separating the two types by the method of fishing colonies. In another case a serum which was supposed to be specific for type C was found to protect also against type A. A type A organism was isolated from one of the type C cultures used in the production of the serum. The indications thus far point to the complete specificity of the different types of antitoxins, and in case such specificity does not appear to exist no definite conclusions can be drawn without first determining whether single-cell isolations from the culture used in producing the antitoxin behave in a manner similar to the original culture.

The method found most productive of results for obtaining single-cell growths consisted in the use of young spore cultures, with transplantation into cooked meat medium. A higher percentage of growths was obtained if spores not more than 24 to 48 hours old were used. At this stage they are still attached to the rods and are not only more readily distinguished but are in a much more vigorous condition than when unattached. The unattached spores are not so readily distinguished, owing to the fact that at times artefacts or specks of foreign material may be mistaken for spores. All of the cultures studied produced spores which in the early stages were so large and conspicuous that there was no question but that single organisms were isolated. Success in obtaining results lies in the use of young cultures, properly prepared pipettes, and suitable culture media. When once the technic is acquired, isolations may be made with comparative ease, and the satisfaction of knowing that such cultures are unquestionably pure well repays one for the time spent in mastering the technic.

The cultures were usually grown in both the cooked meat medium and in one-tenth per cent agar, a vaseline cap being always used to provide better anaerobic conditions, both in the case of the culture from which single-cell isolations were to be made and the culture medium into which the single cell was to be planted. The cap may be melted and the tube tipped so that when inserting the pipette into the tube one may avoid carrying it through the vaseline. The pipette is lowered to the bottom of the tube and the tip containing the organism pressed against the bottom of the tube and broken off.

It was found advantageous to make daily examinations of the cultures from which spores were to be fished and as soon as the spores appeared in the rods to begin making isolations. In some cases spores appeared early. In the case of the cultures obtained

from the limberneck material spores always appeared on the day following inoculation into the meat medium. In some other cases it was necessary to wait for five or six days. For certain of the cultures the meat medium seemed to be most favorable for spore formation, while in others more satisfactory results were obtained with the one-tenth per cent agar. In the following pages more detailed information is given in regard to the various cultures.

Nos. 7 and 9, strains Saunders.—The first work was done with the Saunders culture, the culture which as previously stated varied from the well-known American types A and B both in its cultural behavior and in the kind of toxin produced. The results obtained with this culture are of special interest in that of the three single-cell isolations which grew, only one produced toxin, while the other two failed to produce toxin. It was felt that this observation merited further investigation. The behavior in the meat medium was so similar in the case of the three cultures that it did not seem to be a question of the original culture being contaminated, though it was of course necessary to know whether this was true or not. The study of this phase of the work is presented on page 41. The toxic culture is represented by No. 7 in the series of cultures studied and the nontoxic culture by No. 9.

No. 11, strain No. 117.—The culture from which the single-cell isolations were made was a mixed culture obtained by planting a fly larva from the crop of a limberneck chicken into meat medium after washing in 5 per cent phenol, 20 per cent alcohol, and in sterile water. An attempt was made to isolate the toxic colonies from liver agar shake cultures made from the meat culture but without success. Single-cell isolations were then made directly from the mixed culture. This was accomplished without great difficulty, the characteristic terminal spored organisms appearing very conspicuously in hanging drop preparations. Growths were obtained from three such isolations and two of these proved to be toxic. The results obtained in this case show the advantage of making isolations directly from the original culture under certain circumstances. In mixed cultures containing a large number of organisms which are active gas formers it is not possible to fish the desired colonies except from tubes containing a few colonies. This necessitates making a very large number of tubes. A few characteristic spore-bearing organisms may, on the other hand, be readily distinguished in a hanging drop containing a very large number of other organisms, and these may be separated and fished by the single-cell method.

No. 13, strain No. 121.—The culture from which the single-cell isolations were made was obtained by planting a fly larva received in glycerin without previous heating into a liver broth fermentation tube. Liver agar shake cultures were made on the following day

and from these a characteristic colony was fished into cooked meat medium. This proved to be toxic, and single-cell isolations were made from this culture. Five single-cell isolations grew, and all of these were toxic.

No. 15, strain No. 3421.—This culture was received as an agar shake culture. A transplant was made into cooked meat medium and this was found to be toxic. Liver agar shake cultures were made from this tube for the purpose of obtaining suitable colonies for fishing. Eight colonies were fished into meat medium and five of these failed to grow characteristically—i. e., failed to produce gas—while three showed a characteristic appearance. One of the latter was tested on mice and found to be toxic, while one of the five which did not produce gas was nontoxic. The results show, therefore, that the original culture was not a pure culture. Single-cell isolations were made from the toxic culture. Two isolations which grew proved to be toxic.

No. 23, strain No. 526.—No effort was made to establish the purity of the culture by other means than single-cell isolations from the transplant made into cooked meat medium. Five single-cell isolations were made and four of these grew. Two were tested on mice and found to be toxic.

No. 25, strain No. 487.—Several single-cell isolations were made from a cooked meat medium transplant from the original culture. Two of the three cultures tested were toxic.

The remaining strains were received as supposedly pure cultures. The record of isolations from these is as follows:

No. 1, strain Memphis.—Four single-cell cultures were alike as to growth in cooked meat medium and all were toxic.

No. 3, strain A 79 (Lister Institute culture No. 95).—Eight single-cell cultures were isolated. All were toxic and showed typical growth in cooked meat medium.

No. 17, strain No. 619.—Two single cells were isolated. Both were toxic and showed typical growth in cooked meat medium.

No. 27, Canton strain.—This culture when received showed two kinds of organisms in smears. One had typical type A spores, and the other organism was much smaller, with a terminal spore. Three organisms were fished and grew and two of these were toxic and produced typical growth in cooked meat medium.

No. 5, Nevin strain.—Four single-cell isolations grew with characteristic appearance in cooked meat medium. These were tested on mice and three were found to be toxic. The other culture was nontoxic.

No. 19, strain No. 465.—Four single-cell growths were obtained. All grew characteristically in meat medium and the two cultures tested were toxic for mice.

No. 21, Lister (Institute strain No. 94).—Considerable difficulty was experienced in obtaining growth with single cells of this culture. Growth was not evident for 7 or 8 days in any of the cultures and in some cases several months elapsed before growth appeared. This strain throughout the work seemed to grow less readily in the culture media used than the others. In the beginning it appeared that none of the cultures would grow and consequently a considerable number of single cells were isolated, using both vegetative and spore forms and using for media both the cooked meat and one-tenth per cent agar. Within the course of six or seven months, however, 18 cultures grew. All showed the same appearance in the meat medium and six tested on mice were found to be toxic.

MORPHOLOGY AND SPORE FORMATION. (Pl. I, fig. 1–4.)

Morphology and spore formation were studied both from stained preparations and hanging drop preparations. The isolation of single cells or a few cells in the small drops made use of in the Barber technic afforded a good opportunity for studying the morphology under favorable conditions. This method was often more satisfactory than the stained preparations, for the reason that some of the strains stained with difficulty. This was particularly true of the members of the fourth group. In hanging drops the terminal spored organisms of this group were very conspicuous, the spore appearing as a shining oval body at the end of a comparatively long rod, the spore being only slightly wider than the rod. In these cultures spores appeared very early in the meat medium, always within 24 hours. The spore-bearing organisms in the Lister Institute culture No. 94 resembled these very closely in morphology, though the organisms were more actively motile and the spores appeared somewhat later. Some cells showed spores in a median position, though usually they were terminal and not appreciably wider than the rod. The cultures of the first and second groups showed spores in the early stages which contrasted sharply with those described. In these the spore instead of appearing to be at the end of the rod was in the rod. The whole rod appeared to swell and the spores were two or three times the width of a vegetative organism. At a later stage organisms were sometimes found in which the spore appeared to have become terminal. The type B strains had a tendency to show spores which were terminal or subterminal rather than median but these were different from the spores of the fourth group in that the part not occupied by the spore was usually short. Detached spores in older cultures were very similar in all the cultures, appearing as oval bodies.

In general the young vegetative forms of all the cultures were very similar, appearing as rods which occurred singly in pairs, or

short chains. Occasionally very long chains appeared. The single organisms averaged about 3 to 8 by 0.5 to 0.8 μ in 24 hours, cultured in meat media, the strains of the third and fourth groups being in general longer and thicker than those of the first and second groups.

Smears of 7 to 8 hour cultures when stained by the Gram method were all Gram positive. The cultures in meat medium soon became Gram negative. The strains comprising the fourth group in meat medium stained with considerable difficulty even after 24 hours and often presented a curious granular or barred appearance.

Motility.—The organisms of the first and second groups may be described as being actively motile. No. 21 (Lister Institute culture No. 94) was also definitely motile. The strains in the fourth group, however, contrasted strongly with these. When first studied they seemed to be entirely nonmotile, but in very young preparations, a slight motility was manifest. The difference in degree of motility is probably to be explained by the difference in the number and kind of flagella. In the case of the American type A and B cultures and the Lister Institute culture No. 95 the flagella were very numerous, varying from 15 to 30 or more (pl. 1, fig. 5–6). They were very long and luxuriant in appearance. The flagella of strain No. 21 (Lister Institute strain No. 94) and the fourth group of cultures on the other hand were much finer and shorter and less numerous, the number in the case of culture No. 21 being about 15 to 20 (fig. 7) and in the culture No. 7 of the fourth group about 5 to 15 (fig. 8). It was necessary to use the oil immersion lens to see clearly the flagella in these cultures, while in the American type A and B cultures they were plainly visible with the high-power dry lens. Van Ermengem (1897 *a*, *b*) states that his culture had 4 to 8 very fine peritrichic flagella and that the organisms showed a slight motility. Some of the organisms of the cultures just described had as few as 8 flagella, but the majority had more. Ornstein states that the organism studied by him had 6 to 20 flagella. This organism was identical with the van Ermengem type serologically.

COMPARATIVE CULTURAL STUDY.

Cultural tests were carried out on a number of the ordinary media and also on special media designed to show the saccharolytic and proteolytic activities of the strains studied. Colony formation was determined in glucose agar and liver agar stabs. Growth was also tested in plain broth, glucose broth, and liver broth. A vaseline cap was used on the fluid media to insure good anaerobic conditions, the media being boiled for one-half hour to expel air after the vaseline was added.

Liver media were found to be favorable for the growth of the cultures, particularly for the nonproteolytic strains Nos. 7, 9 11,

13, 15, 23, 25, and 21. The superiority of the liver media over beef infusion media was shown in the comparatively luxuriant growth obtained in this medium contrasted with the scant growth or the failure of growth in media made with beef infusion as the base. The addition of glucose to beef infusion medium was not effective in bringing about active growth, though in one-tenth per cent agar containing 1 per cent of glucose, conditions apparently were favorable for growth, and the glucose was vigorously fermented.

The growth capacity of the various groups of the organisms studied is well illustrated in the attempts which were made to obtain surface growths to be used for making preparations to demonstrate flagella. The strain of type A used, No. 1, and the one of type B, No. 5, grew without difficulty on the surface of liver agar slants, using the pyrogallic acid method. A heavy moist growth was obtained. In contrast to this, a thin diffuse growth was obtained with culture No. 7. No growth was obtained with culture No. 21, but by the addition of digested casein sufficient growth was obtained for making preparations for staining. The addition of the digested casein also produced more luxuriant growths in the case of culture No. 7 than in media without digested casein. The growth was considerably more luxuriant than that of culture No. 21.

The results described thus show a gradation in the growth capacity of the various cultures. The culture No. 21 was the most difficult to grow. This was indicated also by the fact that in many cases growth from single cells was delayed over a period of several months, as described earlier. Culture No. 7 and others of this group in a number of instances also showed a reluctance or failure to grow in certain media, while vigorous growth was practically always obtained with the type A and B cultures Nos. 1, 3, 17, 27, 5, and 19.

Growth in fermentation tubes.—Plain broth fermentation tubes: Type A and B cultures Nos. 1, 3, 17, 27, 5, and 19, comprising the first and second groups, grew well and in some tubes a small gas bubble was formed. The strains in the fourth group, cultures Nos. 7, 9, 11, 13, 15, 23, and 25, showed very slight growth (which was promptly precipitated, or sometimes no growth. Culture No. 21 either showed a small amount of growth or sometimes failed to grow.

One per cent glucose broth fermentation tubes: Luxuriant growth was obtained with the type A and B cultures of the first two groups with the production of 20 to 100 per cent of gas in the course of seven days. A very light flocculent growth occurred in the case of cultures Nos. 7, 9, 11, 13, 15, 23, and 25. Sometimes there was no growth. There was also no production of gas. Culture No. 21 showed a small amount of growth and sometimes a small gas bubble. The growth was promptly precipitated.

Liver broth: Very good growth was obtained with this medium in the case of the cultures Nos. 7, 9, 11, 13, 15, 23, and 25, of the fourth group, which grew with such difficulty in glucose broth. Five to 10 per cent of gas was usually formed in the course of seven days. Culture No. 21 also showed heavier growth than in glucose broth, and considerable gas was formed. Very heavy growth was obtained with the type A and B cultures Nos. 1, 3, 17, 27, 5, and 19, and gas production to the extent of 40 to 90 per cent.

Growth in stab cultures.—In the plain agar and glucose agar stab cultures practically the same variations in the vigor of growth occurred as in the broth cultures. Fairly good growth was however usually obtained with the cultures Nos. 7, 9, 11, 13, 15, 23, and 25, after a period of delay, though occasionally a culture failed to grow. The growth was characterized by fringy projections into the medium, while in the case of the type A and B cultures of the first two groups these were absent. The same appearance was noted in the case of the glucose agar stab growth of these cultures. Much better growth was obtained in these media with the type A and B cultures Nos. 1, 3, 17, 27, 5, 19, which produced a considerable amount of gas in the glucose agar stabs. In the liver agar stabs good growth was obtained in the case of all of the cultures.

Colony formation.—Colony formation was studied in glucose agar and liver agar stabs, successively higher dilutions of the culture being obtained by carrying the same pipette dipped into the culture through a series of tubes. Colonies described by Burke (1919, *a*) as typical were obtained with the type A colonies in glucose agar (discoidal, compact, with definite outline and small opaque knot or "nucleus" at periphery). The type B Nevin strain (No. 5) throughout the work has shown a tendency to form a woolly colony, more closely resembling the colony of *C. tetani*. Strain No. 21 (Lister Institute No. 94) showed a rather fluffy colony with a more dense center. The colonies of the group 4 cultures (Nos. 7, 9, 11, 13, 15, 23, and 25) were very diffuse, without a compact nucleus, and grew less luxuriantly, as indicated by the fact that usually only the first tube or the first two of a series showed growth. In liver agar shake cultures these strains have a tendency to form colonies which more nearly correspond with what has been considered the typical *C. botulinum* colony. The cultures Nos. 1, 3, 17, 27, 5, and 19, on the other hand, often showed a diffuse colony in the liver media. The kind of medium used therefore influences the growth of the colony as does also the moistness and freshness of the medium. As to gas production the type A and B cultures Nos. 1, 3, 17, 27, 5, 19, and culture No. 21 produced a large amount of gas. The remaining cultures for the most part produced no gas or a slight amount of gas. These often showed a tendency to

grow between the tube and the medium and the whole agar stab might be pushed up. Occasionally there was a crack or two in the medium but there was never any pronounced fragmentation as was found in the case of the other cultures although the tube was crowded with colonies.

Action on various protein media (Table I).—The accompanying table shows a summary of the results obtained in the various media used for testing proteolytic activity. In this connection a number of the media used by Kahn in his study of a collection of single cell cultures of anaerobic organisms were employed. The greater part of the tests were carried out at a temperature of 37.5° C. though a few tests were made at room temperature.

TABLE I.—*Cultural reactions in protein media.*

	Cooked meat medium.[1]			Brom Cresol purple milk.				
	Gas (15 days).	Change in appearance of meat (15 days).	Digestion of meat (15 days).	Acid (48 hours).		Gas (12 days).	Precipitation and digestion of casein.	
							48 hours.	12 days.
	Inches.		*Inches.*			*Inches.*		
1. Memphis	4	Dark red, comminuted.	1¼	±		¼	±	++++
3. Lister Institute No. 95.	3¼	.....do.....	1	±		¾	±	++++
17. Boise City	3½	.....do.....	1⅛	±		½	±	++++
29. Canton	2¼	.....do.....	1¼	±		1½	±	++++
5. Nevin	1¾	Very dark red, comminuted.	1½	±		1¼	±	++++
19. No. 465, oats	2	Dark red, comminuted.	1¼	±		¾	±	++++
				72 hours.	15 days.			
21. Lister Institute No. 94.	1⅛	No change	0	±	++	—(4 mos.)	—(4 mos.)	
7. Saunders	1⅜	No change	0	±	±	—(4 mos.)	—(4 mos.)	
9. Saunders, nontoxic.	¾	.....do.....	0	±	++	...do.....	...do.....	
11. No. 117	1	.....do.....	0	±	+	...do.....	...do.....	
13. No. 121	1	.....do.....	0	±	+	...do.....	...do.....	
15. No. 3421	1½	.....do.....	0	±	+	...do.....	...do.....	
23. No. 526	¾	.....do.....	0	±	±	...do.....	...do.....	
25. No. 487	¼	.....do.....	0	±	+++ (coag.)	...do.....	...do.....	

[1] The tubes used were large-sized test tubes 5¾ by ¾ inches. The column of medium was approximately 3 inches deep, of which two-thirds consisted of meat.

TABLE I.—*Cultural reactions in protein media*—Continued.

	Loeffler's serum.[2]					Gelatin.		
	Gas.		Liquefaction.			Growth (24 hours).	Gas.	Liquefaction.
	5 days.	30 days.	24 hours.	30 days.	3 months.			
	Inches.	*Inches.*		*Inches.*				
1. Memphis	½	1½	Drop.	¾	Complete.	++++	+(4 days)	+(4 days)
3. Lister Institute, No. 95.	⅜	1¼	...do...	½	...do...	++++	...do...	Do.
17. Boise City	½	1¾	...do...	½	...do...	++++	...do...	Do.
29. Canton	⅝	1¼	...do...	¾	...do...	++++	...do...	Do.
5. Nevin	½	2½	Drop.	¾	Complete.	++++	+(4 days)	+(4 days)
19. No. 465, oats	¾	1¾	...do...	½	...do...	++++	...do...	Do.
21. Lister Institute, No. 94.	0	1/16 (3 mos.)	0	0	0	+	Bubble...	+11 (da.)
7. Saunders	0	1/16 (30 days)	0	0	Drop.	+	Bubble...	+(11 da.)
9. Saunders, non-toxic.	0	1/16 (3 mos.)	0	0	...do...	−	...do...	Do.
11. No. 117	0	1/16 (3 mos.)	0	0	...do...	−	...do...	Do.
13. No. 121	0	1/16 (3 mos.)	0	0	...do...	++	...do...	Do.
15. No. 3421	0	0	0	0	0	+	...do...	Do.
23. No. 526	0	0	0	0	0	+	...do...	Do.
25. No. 487	0	0	0	0	0	+	...do...	Do.

	Coagulated egg white medium.					Egg fluid medium.		
	Growth.	Gas.	Digestion.			Gas.	Coagulated.	Coagulum digested.
			72 hours.	18 days.	3 mos.			
		Inches.		*Per cent.*				
1. Memphis	++	⅛(3 days)	Slight.	75	Complete.	0 (3 mos.)	+(18 da.)	75 per cent digested (3 mos.).
3. Lister Institute, No. 95.	++	Bubble (3 days).	...do...	80	...do...	...do...	...do...	Do.
17. Boise City	++	⅛(3 days)	...do...	90	...do...	...do...	...do...	50 per cent digested (3 mos.).
27. Canton	++	...do...	...do...	90	...do...	...do...	...do...	25 per cent digested (3 mos.).
			Per ct.					
5. Nevin	++	⅛(3 days).	50	Almost complete.	Complete.	¾(3 days).	+(5 days)	85 per cent digested (3 mos.).
19. No. 465, oats	++	Bubble (3 days).	30	90	...do...	½ (3 days)	+(4 days)	90 per cent digested (3 mos.).
21. Lister Institute, No. 94.	++	0	0	0	0	0(3 mos.).	0	0
7. Saunders	++	0	0	0	0	0(3 mos.).	0	0
9. Saunders, non-toxic.	++	0	0	0	0	...do...	0	0
11. No. 117	++	0	0	0	0	...do...	0	0
13. No. 121	++	0	0	0	0	...do...	0	0
15. No. 3421	++	Bubble (3 days).	0	0	0	...do...	0	0
23. No. 526	++	...do...	0	0	0	...do...	0	0
25. No. 487	++	...do...	0	0	0	...do...	0	0

[2] Small test tubes 5¾ by ½ inch were used. The depth of the medium was approximately 2 inches.

The most striking feature of the cultural tests in the six media used is the vigorous proteolytic action in the case of the strains included in the first two groups and the very slight or negative reaction of the Lister Institute culture No. 94 and the strains in the fourth group consisting of the fly-larvæ and allied cultures. The behavior in the various media will be considered more in detail in the following paragraphs.

Cooked meat medium.—This medium consists of part of chopped beef with 2 parts of water, the reaction being adjusted to pH 8.5. The process of sterilization (autoclaving at 15 pounds pressure for 1½ hours) increases the acidity to about pH 7.0. Growth in this medium was very prompt in the case of all the cultures studied. There was always evidence of growth the day following planting, and in general the appearance in all was very similar. There was marked turbidity and a certain amount of gas production in all the cultures. In 48 hours or sooner there was evidence of proteolytic activity in the cultures of the first group. This was indicated by a darkening of the meat as well as by an appearance of softening or disintegration. This increased in the course of several days, the meat particles becoming more comminuted and darker and the total volume decreasing, due to digestion, so that in the course of about 15 days the amount of undigested meat was reduced to about one-third of the original volume in the case of the type A cultures Nos. 1, 3, 17, and 27. The two type B cultures studied (the Nevin strain and strain No. 465) were somewhat more active proteolytically than the four preceding cultures as indicated by the fact that the volume of the meat was reduced somewhat more and the color was darker. Darkening of the supernatant fluid was present in all. Blackening in these cultures was not constant though it did occur often. The appearance of the meat in the remaining cultures, Lister Institute culture No. 94 and cultures Nos. 7, 9, 11, 13, 15, 23, and 25, contrasted strongly with those just described. There was no evidence of proteolysis as indicated by change of color or softening or disintegration of the meat in 15 days. At the end of four months there was no reduction in the volume of the meat in the Lister Institute No. 94 culture, while in the case of the others there was some indication of this—about 15 to 20 per cent—though there was still no apparent evidence of disintegration of the meat particle or change in color. The amount of gas produced by the cultures of the first two groups was greater than in the others. This amounted to 3 or 4 inches in 15 days, while in the remaining cultures it was about 1 to 1½ inches. The strongly proteolytic cultures remained turbid for a considerable period, 5 to 7 days, while in the nonproteolytic cultures in which the growth became flocculent there was clearing after about 48 hours incubation.

Brom-cresol purple milk.—This medium was made up by the addition of 1 c. c. of a 5 per cent alcoholic solution of brom-cresol purple

to 1,000 c. c. of milk. Group 1 and 2 cultures, Nos. 1, 3, 17, 27, 5, 19, showed a slight change in the color of the medium within 24 hours; after 48 hours there was evidence of a change in the reaction and beginning precipitation of the casein. This was probably more marked in the type B cultures than the type A cultures. In three days the A cultures showed marked liquefaction of the curd, while the Nevin strain showed almost complete liquefaction. All of these cultures showed practically complete digestion of the casein in about 12 days. The undigested part was in the form of loose fluffy material, part of which was precipitated at the bottom of the tube and part of which was at the surface of the fluid. The fluid portion varied in color from amber to brown, the type B cultures showing in general a darker color. A certain amount of gas was produced in all of these cultures, varying from ½ to 1½ inches.

Culture No. 21 (Lister Institute culture No. 94) and the remaining cultures, Nos. 7, 9, 11, 13, 15, 23, and 25, on the other hand, showed slight reactions. A faintly acid reaction was present in two or three days. This gradually became more marked, and in the course of 15 days sufficient acid had been produced to cause coagulation of the casein in culture No. 25. No. 21 (Lister Institute culture No. 94) and No. 9 also showed a decidedly acid reaction in 15 days. At the end of four months there was some evidence of increased acidity, several cultures showing a rather marked acidity, including cultures Nos. 9 and 23, though neither had enough acid to coagulate the casein. No gas was formed in any of these cultures.

Loeffler's serum medium.—This medium consisted of horse serum in the proportion of three parts to one part of 1 per cent glucose broth. The medium was made in the form of stabs and overlaid with vaseline. Groups 1 and 2, cultures Nos. 1, 3, 17, 27 (type A), 5, 19 (type B), acted promptly on this medium. A slight liquefaction with darkening of the coagulated serum was present in 48 hours. In the course of four or five days the digestion has progressed to the extent that in the A cultures about one-fourth of the serum was liquefied and in the B cultures about one-half. About one-half to 1 inch of gas was formed in 5 days. In 30 days the serum was digested to the extent of one-half to three-fourths of the total volume, the fluid being brown and perfectly clear. The serum was completely liquefied except for a small amount of black residue in the bottom of the tubes in about 3 months, the amount of gas varying from 1 to 2½ inches. No. 21 (Lister Institute culture No. 94) and cultures Nos. 7, 9, 11, 13, 15, 23, and 25 of the fourth group showed no evidence of change for about three months except that in the case of culture No. 7 the vaseline cup was raised about one-sixteenth inch in 30 days. In the course of three months a drop of fluid was present in cultures Nos. 7, 9, 11, and 13. The vaseline cap was raised to the extent of one-sixteenth to one-eighth inch in cultures Nos. 9, 11, 13, and 21.

Gelatin:—Liquefaction of gelatine was determined by planting and incubating at 37.5° C. until there was evidence of good growth and then placing at cold room temperature. Prompt liquefaction with gas production took place in the type A cultures, Nos. 1, 3, 17, 27 and type B 5 and 19. Some difficulty was experienced in obtaining growth in the case of culture No. 21 (Lister Institute culture No. 94) and cultures Nos. 7, 9, 11, 13, 15, 23, and 25. A slight amount of flocculent growth occurred after two or three days' incubation, usually accompanied by a small amount of gas production. The growth was soon precipitated. After an incubation period of 11 days the tubes were placed in the cold room. All the cultures were found to have brought about liquefaction.

Coagulated egg white medium:—This consisted of ordinary beef infusion broth adjusted to a reaction of pH 7.6, with a piece of coagulated egg white in the bottom of the tube. Type A cultures Nos. 1, 3, 17, and 27, produced a heavy growth with some gas. In the course of three days the egg white showed evidence of digestion, the edges becoming rounded off, the egg white more transparent and reduced in size. The digestion was almost complete in 15 days and at the end of three months there was no evidence of the egg white except a small amount of flocculent precipitate in the bottom of the tube. Type B cultures 5 and 19 showed about 50 per cent of digestion of the egg white in 3 days and almost complete in 18 days. Culture No. 21 (Lister Institute No. 94) and Nos. 7, 9, 11, 13, 15, 23, and 25, on the other hand, showed no change in appearance of the egg white at the end of three months. The growth was fairly luxuriant in the beginning and in all except No. 21 presented a flocculent appearance. This was soon precipitated in the bottom of the tube. A small amount of gas was formed in the first group of cultures described, and in the second occasionally a bubble of gas was seen.

Egg fluid medium.—This medium consisted of the yolk of one egg and the whites of two made up to 500 c. c. with tap water and adjusted to a reaction of pH 7.6. None of the cultures gave evidence of growth in this medium for two or three days. At this time the type B cultures 5 and 19 showed some gas ($\frac{1}{2}$–$\frac{3}{4}$ inches). In 5 days the A cultures showed evidence of growth by a whitening of the medium. Type B cultures 5 and 19 at this time showed some curdling of the egg. The type A cultures showed curdling in about 15 days. The curd was slowly digested and at the end of 4 months a portion which still retained its cylindrical form as described by Kahn remained undigested. No gas was produced by the A cultures. Type B cultures Nos. 5 and 19 at the end of 4 months showed more complete digestion, the undigested portion appearing as flocculent material on the surface of the clear fluid. The re-

maining cultures, Nos. 21, 7, 9, 11, 13, 15, 23, and 25, showed no evidence of curdling or digestion of the egg and no gas production.

Summarizing the results described in the six media used to test the proteolytic activity of the organisms studied, it may be stated that there is a distinct line of demarcation between the first two groups of cultures and the second two groups—the first two including the three strains of type A and the two strains of type B and the second two including the type B strain No. 21 (Lister Institute culture No. 94) and the seven strains Nos. 7, 9, 11, 13, 15, 23, and 25. The members of the first two groups were strongly proteolytic in all the media used, while the latter two groups gave evidence of no proteolytic activity or of very feeble action. The results obtained with the former group agree in general with those obtained by Kahn in the study of one single-cell strain of type A. It may be said in general that the group 2, type B, cultures were somewhat more actively proteolytic than the type A cultures, though it is doubtful whether a differentiation would be possible on this basis. The difference was one of degree and not of kind, the action usually being somewhat more rapid and pronounced in the case of the B than of the A strains. The strains constituting groups 3 and 4 were inactive and produced slight changes or no change whatever in the various media used. The liquefaction of gelatin and the drop of fluid contained in some of the Loeffler serum cultures are evidences of a certain proteolytic activity. The reduction in the volume of the meat in the cooked meat medium in the case of cultures Nos. 7, 9, 11, 13, 15, 23, and 25 may also be evidence of a slight proteolytic activity. This reduction was not noted in the case of culture 21 (Lister Institute culture No. 94,), which might therefore be considered the least proteolytic of all, or if not this, at least the most fastidious in its growth requirements. This reluctance to grow has been noted also in other connections. There was, however, no appreciable difference in the hydrogen-ion concentration of 7-day meat cultures of the Lister Institute culture and several strains among group 4 (7, 9, 11, 13, 15, 23, and 25) all having a pH value of 6.5 to 6.7, which would point to the fact that there was not appreciably more digestion of meat in the one case than the other. That there may be a considerable difference in the hydrogen-ion concentration of the cultures which are certainly proteolytic and those which are nonproteolytic is shown by the fact that the hydrogen-ion concentration of the cultures, types A and B, Nos. 1, 3, and 5, varied from 6.8 to 7.2 after 7 days' incubation, while that of 7, 11, and 15 was pH 6.0. The matter of the significance of proteolysis will be considered more in detail later.

Action on carbohydrates and related substances.—Varying results have been obtained in testing the fermentative properties of these

organisms. Van Ermengem (1897) in his earliest publications states that abundant gas is produced in glucose media and only a very little is formed from saccharose and lactose. In his latest publication (1912) he states that there is no fermentation of lactose and saccharose. The Medical Research Committee of Great Britain (1919), who describe the Lister Institute culture No. 94, report the following substances fermented: Glycerin, glucose, maltose, lactose, starch. The following substances were not fermented: Inulin, mannite, dulcite, salicin.

Regarding the fermentation reactions of the American types A and B we find also little agreement. Thom, Edmonson, and Giltner state that the Boise City strain when grown in 1 per cent sugar yeast water medium produced no gas but slight acidity, as indicated by litmus indicator in xylose, galactose, mannite, maltose, inulin, trehalose, rhamnose, and glycerin. There was no acid nor gas formation in dulcite media. Glucose, lactose, and saccharose were fermented, but with gas formation only in glucose.

Reddish found that toxic isolations from a number of cultures not known to be certainly pure fermented glucose, levulose, maltose, lactose, and saccharose with gas production. Armstrong, Story, and Scott report that the Canton, Ohio, strain fermented glucose, saccharose, lactose, and mannite with gas and acid production. Kendall, Day, and Walker who studied 6 cultures found that all fermented glucose, maltose, and glycerin. Starch was attacked by two of the cultures and saccharose was slowly decomposed by two. Lactose was not acted on by any. Nevin states type B isolated from cottage cheese fermented the following carbohydrates with production of gas: Dextrose, galactose, levulose, lactose, saccharose, maltose, mannite, dextrin, inulin, and glycerin. Kahn states that his strain (type A) attacked only the monosaccharides, though his table shows that gas and acid reaction was produced in melezitose and glycerin. Variations in the media used, differences in the methods of sterilization, and the question of purity of the cultures in some cases, as well as the purity of the carbohydrates used, are factors to be considered in explaining the different results obtained.

The results obtained by the writer are shown below. The following carbohydrates and related substances were used in this work: Dextrose, levulose, galactose, saccharose, lactose, maltose, raffinose, dextrin, inulin, glycerin, adonite, dulcite, mannite, xylose, arabinose, inosite, and salicin.[4] The base medium consisted of one-tenth per cent agar, made with sugar-free broth and containing 1 per cent digested

[4] The following substances were Pfanstiehl preparations: Levulose, galactose, saccharose, lactose, maltose, raffinose, dextrin, adonite, dulcite, mannite, xylose, arabinose, inosite, salicin. The dextrose was from the J. T. Baker Chemical Co., the inulin from Merck's, the glycerin from James Good (Inc)., and the rhamnose from the Eastman Kodak Co.

casein. This was adjusted to a reaction of pH 7.5, though it was found that there was some change of reaction brought about by the addition of the carbohydrate substance. The medium was tubed in medium-sized culture tubes, and a sufficient amount of a 10 per cent solution of each of the various carbohydrates or related substances added so that a final concentration of 1 per cent was obtained. The sugar solutions were heated in the Arnold for a period of 20 minutes. After adding the carbohydrate solution to the sugar-free one-tenth per cent agar, the tube was overlaid with sterile vaseline and heated in the Arnold for 20 minutes.

Inoculations were made by means of Pasteur pipettes, the cultures having previously been grown in cooked meat media for 18 hours. The cultures were incubated at 37.5° C. for a period of 4 days. Control tests consisted in determining the reaction of the various uninoculated carbohydrate media after incubation at the same temperature and for the same length of time as the inoculated tubes, and also the determination of change in reaction in tubes of the medium to which no carbohydrate had been added, and which had been inoculated with the various cultures. The results are presented in the following table (Table II):

TABLE II.—*Fermentation of carbohydrates and related substances.*

	Dextrose.	Levulose.	Galactose.	Saccharose.	Lactose.	Maltose.	Raffinose.	Dextrin.	Inulin.	Glycerin.	Adonite.	Dulcite.	Mannite.	Xylose.	Arabinose.	Rhamnose.	Inosite.	Salicin.	No sugar.
1. Memphis.....	5.6 +++*1.8*	6.0 +++*2.0*	7.0 ++*b*	7.1 ++*b*	7.0 +++*b*	5.6 +++*1.8*	7.0 +++*b*	6.6 ++*b*	7.1 +++*b*	6.2 ++*1.9*	7.1 +++*b*	7.1 +++*b*	7.1 +++*b*	7.1 +++*b*	7.1 ++*b*	7.1 ++*b*	7.1 ++*b*	6.0 +++*1.2*	7.1 ++*b*
3. Lister Institute No. 95.	5.8 +++*1.5*	6.0 +++*0.6*	7.0 ++*b*	7.1 +++*b*	7.1 +++*b*	5.4 +++*1.5*	7.1 +++*b*	6.5 ++*b*	7.1 +++*b*	6.4 ++*1.2*	7.1 +++*b*	7.1 +++*b*	7.1 ++*b*	7.1 +++*b*	7.1 ++*b*	7.1 ++*b*	7.1 ++*b*	6.0 +++*0.7*	7.1 ++*b*
17. Boise City...	6.0 +++*2.5*	6.3 +++*b*	7.1 ++*b*	7.1 ++*b*	7.1 ++*b*	5.6 +++*1.2*	7.1 ++*b*	6.6 ++*b*	7.1 ++*b*	6.4 ++*1.7*	7.1 ++*b*	7.3 +*b*	7.1 ++*b*	7.1 ++*b*	7.1 +*b*	7.1 ++*b*	7.1 ++*b*	6.0 ++*1.2*	7.1 ++*b*
27. Canton.....	5.8 +++*2.0*	6.2 +++*0.1*	7.1 ++*b*	7.1 ++*b*	7.1 ++*b*	5.6 +++*1.8*	7.1 ++*b*	6.5 ++*b*	7.1 ++*b*	6.4 ++*1.9*	7.1 ++*b*	7.1 ++*b*	7.1 +++*b*	7.1 ++*b*	7.0 ++*b*	7.1 ++*b*	7.1 ++*b*	6.0 +++*0.9*	7.1 ++*b*
5. Nevin.....	5.6 +++*2.0*	5.4 +++*1.5*	7.0 +++*b*	7.0 +++*b*	7.0 +++*b*	5.8 ++*1.2*	7.0 +++*b*	6.6 +++*0.1*	7.0 +++*b*	6.2 +++*1.7*	7.0 +++*b*	7.0 +++*b*	7.0 +++*0.1*	7.0 +++*b*	7.0 +++*b*	7.0 +++*b*	7.0 ++*b*	7.0 +++*0.1*	7.0 +++*b*
19. No. 465 (oats)	5.8 +++*1.9*	6.7 +++*b*	7.0 +++*b*	7.0 +++*b*	7.0 ++*b*	5.8 +++*0.9*	7.0 ++*b*	6.7 +++*b*	7.0 +++*b*	6.3 +++*0.6*	7.0 +++*b*	7.0 +++*b*	7.0 +++*b*	7.0 +++*b*	7.0 ++*b*	7.0 +++*b*	7.0 ++*b*	6.6 +++*0.1*	7.0 +++*b*
21. Lister Institute No. 94.	5.4 +++*1.5*	5.2 +++*0.5*	7.0 +*b*	7.3 ±	7.4 ±	5.4 +++*2.0*	7.4 +*b*	6.0 +++*2.1*	7.3 ±	6.4 +++*b*	6.0 +++*1.3*	7.4 ±	7.4 ±	7.2 ±	7.2 ±	7.2 +*b*	5.8 +++*b*	7.4 ±	7.4 ±
7. Saunders.....	5.4 +++*b*	6.3 ++*b*	6.1 +++*2.0*	7.4 ±	7.4 ±	5.8 +++*1.7*	7.4 ±	7.1 +*b*	7.3 ±	6.2 +++*b*	7.4 ±	7.4 ±	7.4 ±	7.2 ±	7.2 ±	7.3 ±	5.5 +++*b*	7.4 ±	7.4 ±
9. Saunders (nontoxic)	5.4 +++*b*	6.4 ++*b*	6.0 +++*2.0*	7.2 ±	7.3 ±	5.8 +++*1.8*	7.4 ±	7.1 +*b*	7.3 ±	6.2 +++*b*	7.4 ±	7.4 ±	7.4 ±	7.2 ±	7.2 ±	7.3 ±	5.4 +++*b*	7.4 ±	7.4 ±
11. No. 117.....	5.4 +++*b*	6.4 ++*b*	6.0 +++*0.5*	7.2 ±	7.3 ±	6.0 +++*1.9*	7.4 ±	7.1 +*b*	7.3 ±	6.4 ++*b*	7.4 ±	7.4 ±	7.4 ±	7.2 ±	7.2 ±	7.3 ±	5.4 +++*0.3*	7.4 ±	7.4 ±
13. No. 121.....	5.6 +++*1.3*	6.6 ++*0.1*	6.1 +++*1.5*	7.2 ±	7.4 ±	5.8 +++*1.5*	7.4 ±	7.1 +*b*	7.3 ±	6.4 +++*0.1*	7.4 ±	7.4 ±	7.4 ±	7.2 ±	7.2 ±	7.3 ±	5.4 +++*0.3*	7.4 ±	7.4 ±
15. No. 3421.....	5.4 +++*1.1*	6.6 ++*0.1*	6.1 +++*0.6*	7.2 ±	7.4 ±	5.8 +++*2.0*	7.4 ±	7.1 +*b*	7.3 ±	6.4 ++*b*	7.4 ±	7.4 ±	7.4 ±	7.2 ±	7.2 ±	7.3 ±	5.4 +++*0.3*	7.4 ±	7.4 ±

23. No. 526.....	5.6	6.6	6.1	7.3	7.4	6.0	7.4	7.1	7.3	6.4	7.4	7.4	7.4	7.2	7.2	7.3	5.5	7.4	7.4
	+++0.8	++0.1	+++0.4	±	±	+++2.3	±	+b	±	++b	±	±	±	±	±	±	+++b	±	±
25. No. 487.....	5.3	6.5	6.0	7.2	7.4	5.8	7.4	7.1	7.3	6.4	7.4	7.4	7.4	7.2	7.2	7.3	5.5	7.4	7.4
	+++b	++b	+++0.9	±	±	+++2.5	±	+b	±	+++b	±	±	±	±	±	±	+++b	±	±
Control (not inoculated)....	7.3	7.3	7.4	7.4	7.5	7.4	7.4	7.4	7.3	7.5	7.4	7.4	7.5	7.3	7.3	7.4	7.4	7.5	7.5

The upper row of figures indicates the reaction in terms of pH after 4 days' incubation.
Figures in the lower row indicate inches of gas. *b* signifies a bubble of gas.
The vigor of growth is indicated by +++ for heavy growth; ++ for moderately heavy growth, + for fair growth, ± for slight growth.

In interpreting the results of this test it is necessary to consider the reactions both of the uninoculated controls of the base medium after the addition of the carbohydrate or related substance and the reactions of the media without the addition of sugar inoculated with the various cultures. The reaction of the medium before the addition of the fermenting substances was pH 7.5. After the addition of the various sugars and other substances the reactions changed in some cases, as indicated in the lowest row of figures. The results with the inoculated controls of medium to which no sugar was added indicate that the medium was probably not entirely sugar free, as the reaction became somewhat more acid in the case of a number of the cultures. In determining whether utilization of the test substance had taken place, any increase in hydrogen-ion concentration, which is less than that of either of the controls, therefore is not to be considered. A difference of 0.2 in pH value is also probably not sufficient to consider. On this basis the results obtained were as follows:

The type A cultures Nos. 1, 3, 17, and 27 (Group 1) fermented dextrose, levulose, maltose, dextrin, glycerin, and salicin, the highest acidity being attained in dextrose and maltose. The fermentation of dextrin was not very pronounced, though very good growth was evident. The reaction on the test substances galactose, saccharose, lactose, raffinose, inulin, adonite, dulcite, mannite, xylose, arabinose, rhamnose, and inosite may be considered negative.

The type B cultures Nos. 5 and 19 (Group 2) fermented the same substances in general as the type A cultures with the exception of salicin, which was not fermented by strain No. 5. The fermentation of levulose was also considerably more in the case of culture No. 5 than with No. 19. As in the case of the type A strains, the most decided reactions occurred in dextrose and maltose, except that levulose was very actively fermented by No. 5, the Nevin strain.

The Lister Institute strain No. 94 (No. 21) fermented dextrose, levulose, maltose, dextrin, glycerin, adonite, and inosite. The results agree fairly well with those reported for this culture in the work of the Medical Research Committee of Great Britain (1919). In that report, lactose is included among the substances fermented.

The remaining cultures, Nos. 7, 9, 11, 13, 15, 23, and 25 of the fourth group, fermented actively dextrose, galactose, maltose, inosite, levulose, and glycerin. There was slight utilization of dextrin. The remaining substances, saccharose, lactose, raffinose, inulin, adonite, dulcite, mannite, xylose, arabinose, rhamnose, and salicin may be considered negative. Growth was slight in the medium when the carbohydrate was not utilized, and it is possible that with a medium sufficiently favorable so that the growth of the culture could be established other carbohydrates might have been utilized.

The most outstanding differential feature in the fermentation reactions in the various groups is the active fermentation of galactose and inosite by the members of the fourth group. No. 21 (Lister Institute strain No. 94) is characterized by its rather active fermentation of inosite and by its definite action on adonite. The active fermentation of salicin is characteristic of the type A cultures.

IMMUNOLOGICAL RELATIONS.

TYPE OF TOXIN PRODUCED.

The type of toxin produced by the various cultures was determined by testing against antitoxins of known type. The occurrence of two groups of organisms in this country producing symptoms of botulism in man and animals, but which elaborated toxins which were not interchangeable by the antitoxins, was first recognized by Dickson in 1917. Burke studied 12 strains of these organisms and by means of toxin-antitoxin tests divided them into two groups. The group designated as "type A" included most of the strains which were concerned in human outbreaks of botulism, while the group designated as "type B" included the Nevin strain and several strains isolated by Graham in his work on forage poisoning. All the strains isolated up to the present time in human outbreaks of botulism in this country have fallen into one or the other of these "types."

As the result of investigations carried out by the writer on fly larvæ (*Lucilia cæsar*), supplied to the Hygienic Laboratory by Dr. E. W. Saunders, who believed them concerned in the causation of poliomyelitis, it was shown that another "type" of organism producing botulism in animals was present. This organism was isolated in pure culture as described and it was found that the toxin produced was neutralized by neither type A nor type B antitoxin. An antitoxin was subsequently produced by inoculating goats with the new toxin and this antitoxin failed to neutralize any of the strains except those of the fourth group. These findings justified the establishing of a new "type" of organism producing botulism in animals, and it was accordingly designated as "type C."

In order to determine the type of toxin produced by any culture, it is necessary to use antitoxins which are known to be specific. A number of A and B toxins received for test were found to contain antitoxin against types other than those appearing on the labels. This would either be due to the fact that horses which had been used for the production of one particular type of antitoxin might later have been inoculated with toxin of another type, or that the horse may have been injected with toxin from a mixed culture (i. e., one containing both A and B strains). The A and B antitoxins used in the typing tests were therefore checked against both types of toxin and shown to be strictly specific. The type C antitoxin produced at

the Hygienic Laboratory was known to be specific, as the goats used for the production of the antitoxin had not previously been used for type A or B antitoxin and the cultures were known to be pure. The tests for specificity of this antitoxin are given in detail on page 46.

The type A antitoxin used (JS4) was tested against type B toxin as follows:

Guinea pig.	Amount of type B toxin.	Approx. M. L. D's.	Amount of type A antitoxin.	Symptoms.	Death.
2322	0.00000057	1	0.01	17 hours	42 hours.
2323	.0000057	10	.01	.....do	Do.
2324	.000057	100	.01		16 hours.

This test shows that the antitoxin does not neutralize even one minimal lethal dose of type B toxin.

The test for potency against type A toxin was as follows:

Guinea pig.	Amount of type A toxin.	Approx. M. L. D's.	Amount of type A antitoxin.	Tested for units.	Symptoms.	Death.
2276	0.00026	100	0.00021	480	27 hours	91 hours.
2277	.00026	100	.00025	400	None	Survived.
2278	.00026	100	.00031	320	None	Do.
2279	.00026	100	.0004	250	None	Do.

The potency of the antitoxin was therefore over 400 units per c. c.

The type B antitoxin used (IN) was tested against type A toxin as follows:

Guinea pig.	Amount of type A toxin.	Approx. M. L. D's.	Amount of type B antitoxin.	Symptoms.	Death.
			c. c.		
2336	0.00026	100	0.01		17 hours.
2337	.000026	10	.01	18 hours	44 hours.
2338	.0000026	1	.01	68 hours	5 days 17 hours.

No protection was afforded against type A toxin.

The tests of this antitoxin against type B toxin indicated a potency of 60 units per c. c.

The antitoxin tests used to determine the type of the various cultures studied are described below. The tests were made by inoculating mice intraperitoneally with the antitoxin and culture. These were given separately, the antitoxin being administered about 15 minutes before the toxin.

The Canton and Boise City strains, Nos. 17 and 27, are the well-known type A strains isolated from olives, the former isolated and described by Armstrong, Story, and Scott, and the latter by Thom,

Edmondson, and Giltner. The Nevin strain is the recognized type B culture.

The following are the results of the tests made to verify or to determine the type of the single-cell cultures used in this study.

NO. 17 (BOISE CITY).

Mouse.		Symptoms.	Death.
1377	0.2 c. c. culture	2 hours	8 hours.
1378	0.2 c. c. culture, plus 100 units type A antitoxin JS4	None	Survived.

NO. 27 (CANTON).

1767	0.2 c. c. culture	1 hour	3 hours.
1765	0.2 c. c. culture, plus 100 units type A antitoxin JS4	None	Survived.

NO. 5 (NEVIN).

2647	0.1 c. c. culture plus 200 units type A antitoxin JS4	2 hours	3½ hours.
2650	0.1 c. c. culture plus 30 units type B antitoxin IN	None	Survived.
2653	0.1 c. c. culture plus 50 units type C antitoxin 295	2 hours	3 hours.
2656	0.1 c. c. no antitoxin	do	Do.

The Memphis strain (No. 1), isolated by the writer from olives concerned in the Memphis (Tenn.) outbreak, was identified as a type A strain at the time of isolation. The tests with the single-cell culture used were as follows:

Mouse.		Symptoms.	Death.
2646	0.1 c. c. culture plus 200 units type A antitoxin JS4	None	Survived.
2649	0.1 c. c. culture plus 30 units type B antitoxin IN	1 hour	3 hours.
2652	0.1 c. c. culture plus 50 units type C antitoxin 295	do	Do.
2655	0.1 c. c. culture no antitoxin	do	4 hours.

Strain No. 3 (Lister Institute culture No. 95) was tested a number of times to determine with certainty the type, since this culture has been designated as a type B culture (Meyer and Dubovsky, 1922, *b*). The following is a test with a transplant of a culture received from Doctor Meyer:

Mouse.		Symptoms.	Death.
1762	0.2 c. c. culture plus 200 units type A antitoxin JS4	None	Survived.
1763	0.2 c. c. culture plus 30 units type B antitoxin IN	1½ hours	4 hours.
1764	0.2 c. c. culture no antitoxin	1½ hours	4 hours.

The single-cell culture used was tested as follows:

Mouse.		Symptoms.	Death.
1638	0.2 c. c. culture plus 200 units type A antitoxin JS4	None	Survived.
1639	0.2 c. c. culture plus 30 units type B antitoxin IN	1½ hours	4 hours.
1640	0.2 c. c. culture plus 10 units type C antitoxin	1½ hours	4 hours.
1641	0.2 c. c. culture no antitoxin	1½ hours	5 hours.

A transplant of this culture was received directly from the Lister Institute, labeled B 95, and this was tested with the following results:

Mouse.		Symptoms.	Death.
1774	0.2 c. c. culture plus 200 units type A antitoxin JS4	None	Survived.
1775	0.2 c. c. culture plus 30 units type B antitoxin IN	4 hours	5½ hours.
1776	0.2 c. c. culture plus 10 to 25 units type C antitoxin 2070	22 hours	92 hours.[1]
1777	0.2 c. c. culture no antitoxin	4 hours	7 hours.

[1] Serum 2070 was later found to contain some type A antitoxin.

The results all show that the culture is a type A culture.

Culture 465. This culture was received from Doctor Graham and found to be a type B culture. The single-cell culture isolated from it was tested as follows:

Guinea pig.		Death.
1421	0.1 c. c. culture	10 hours.
1422	0.1 c. c. culture plus 400 units type A antitoxin JS4	10 hours.
1423	0.1 c. c. culture plus 60 units type B antitoxin IN	Survived.

The Lister Institute culture No. 94 (No. 21) was received as a transplant from a culture obtained by Doctor Reddish and later directly from the Lister Institute. A number of tests were necessary to establish with certainty the type of this culture also. The label indicated a type A culture and the appearance of growth in meat medium (nonproteolytic) indicated a type C culture. The supernatant fluid of the culture as received from the Lister Institute was tested as follows:

Mouse.		Symptoms.	Death.
1756	0.2 c. c. culture plus 200 units type A antitoxin JS4	6½ hours	22 hours.
1757	0.2 c. c. culture plus 30 units type B antitoxin IN	None	Survived.
1758	0.2 c. c. culture no antitoxin	6½ hours	22 hours.

The culture received from Doctor Reddish had previously been tested with the following results:

Guinea pig.		Symptoms.	Death.
1424	1 c.c.culture	44 hours	56 hours.
1426	1 c. c. culture plus 400 units type A antitoxin JS4		18 hours.
1428	1 c. c. culture plus 60 units type B antitoxin IN	None	Survived.

Another test was carried out on mice with the same culture as follows:

Mouse.		Symptoms.	Death.
1356	0.2 c. c. culture plus 200 units type A antitoxin JS4	20 hours	24 hours.
1357	0.2 c. c. culture plus Polyvalent antitoxin 14 K; 630 units type A; 370 units type B	None	Survived.
1358	0.2 c. c. culture plus 15 units type C antitoxin		7 hours.
1359	0·2 c.c . culture no antitoxin	20 hours	43 hours.

Some of the single-cell cultures isolated from this strain were tested as follows:

Culture *a*, Feeding test on guinea pigs:

Guinea pig.	Culture.	Antitoxin.	Symptoms.	Death.
1538	0.5 c. c. 72-hour culture..	No antitoxin	46 hours..	74 hours.
1539	0.1 c. c. 72-hour culture..	....do	74 hours..	5 days 7 hours.
1540	0.01 c. c. 72-hour culture.	....do	None.....	Survived.
1541	0.5 c. c. 72 hour culture..	30 units type B antitoxin IN	.....	Do,
1542	0.1 c. c. 72-hour culture..	....do	None.....	Do.
1543	0.01 c. c. 72-hour culture.	....do	do.....	Do.

Culture *a*, Intraperitoneal inoculation of mice:

Mouse.		Symptoms.	Death.
2630	0.2 c. c. culture plus 200 units type A antitoxin JS4	3 hours....	5 hours.
2632	0.2 c. c. culture plus 30 units type B antitoxin IN	None.....	Survived.
2638	0.2 c. c. culture plus 50 units type C antitoxin 295	3 hours....	4 hours.
2640	0.2 c. c. culture, no antitoxin	...do.....	Do.

Similar results were obtained with the single-cell culture *c*.

Four other single-cell cultures, *b*, *d*, *e*, *f*, were tested with the type B antitoxin and in all cases the mice were protected.

The tests carried out all indicate a type B culture as regards toxin production, though the cultural behavior differed from that of the other type B strains, No. 5 and No. 19, as described.

The remaining 10 single-cell cultures isolated were not tested as to type but all showed a similar growth in the cooked meat medium and in all probability were type B.

The antitoxin for the type C cultures was produced by inoculating goats with the toxin of the culture originally isolated, the Saunders culture, and later, when the original culture failed to produce sufficiently strong toxin, with culture No. 13 (121).

The test made with the antitoxin produced by inoculation of the toxin of the Saunders' culture against the toxin of culture No. 13 is as follows:

Mouse.		Symptoms.	Death.
1144	0.2 c. c. culture, plus 0.5 c. c. type C antitoxin (25 units per c. c)	None.....	Survived.
1145	0.2 c. c. culture, no antitoxin	2 hours...	4 hours.

The results show therefore that the antitoxin protects against culture No. 13 as well as against the culture first used for immunizing.

The following results were obtained in testing on mice the type C cultures against Polyvalent antitoxin 14 K (630 units of Type A and 370 units type B antitoxin) and type C antitoxin 296 (75–100 units).

Single-cell culture No.	Type C antitoxin.		Polyvalent (A and B) antitoxin.		No antitoxin.	
	Symptoms.	Death.	Symptoms.	Death.	Symptoms.	Death.
7	None	Survived	6 hours	7 hours	5 hours	6 hours.
11	do	do	do	8 hours		2 hours.
13	do	do	2 hours	4 hours	2 hours	5 hours.
15	do	do	do	3 hours		2 hours.
23	do	do	do	4 hours	2 hours	5 hours.
25	do	do	do	3 hours	do	Do.

CLASSIFICATION AND NOMENCLATURE.

On the basis of the antitoxin tests the cultures may therefore be grouped as follows:

Type A:
- No. 1, Memphis culture.
- No. 3, Lister Institute culture No. 95.
- No. 17, Boise City culture.
- No. 27, Canton culture.

Type B:
- No. 5, Nevin culture.
- No. 19, culture No. 465 (oats).
- No. 21, Lister Institute culture No. 94.

Type C:
- No. 7, culture Saunders (fly larvæ).
- No. 11, culture No. 117 (fly larvæ).
- No. 13, culture No. 121 (fly larvæ).
- No. 15, culture No. 3421 (limberneck chicken).
- No. 23, culture No. 526 (stomach contents of horse).
- No. 25, culture No. 487 (limberneck chicken).

The cultural characteristics of the members of each of the toxin groups are consistent, with the exception of the type B group, in which No. 5 and No. 19 are proteolytic and No. 21 nonproteolytic. The relationship may be presented thus (Table III):

TABLE III.—*Classification by action on proteins and type of toxin.*

Type of toxin.	Action on proteins.		
	Nonproteolytic or feebly proteolytic.	Proteolytic.	
Type A		1, Memphis strain 3, Lister Institute strain No. 95 17, Boise City strain 27, Canton strain	Neutralized by specific type A antitoxin.
Type B	21, Lister Institute strain No. 94		
		5, Nevin strain 19, strain No. 465	Neutralized by specific type B antitoxin.
Type C	7, Saunders strain (fly larvæ) 11, strain No. 117 (fly larvæ) 13, strain No. 121 (fly larvæ) 15, strain No. 3421 (limberneck chicken) 23, strain No. 526 (stomach contents of horse). 25, strain No. 487 (limberneck chicken).		Neutralized by specific type C antitoxin.

In reviewing the data presented regarding the cultural characteristics of the strains studied and the types of toxin produced the question arises as to the nomenclature and classification of the members of this group of organisms. If the nonproteolytic strains all produced a type of toxin which differed from that produced by all the proteolytic strains, it would appear logical to separate the nonproteolytic from the proteolytic strains. The fact that a nonproteolytic and a proteolytic strain (No. 21 and No. 5) produce the same type of toxin complicates the situation. It is debatable whether on the one hand the elaboration of different types of toxins which produce the same effects on animals or on the other hand the presence or absence of a proteolytic enzyme and certain morphological differences should be considered as of first importance in species characterization. The classifications of anaerobes proposed emphasize the physiological or cultural behavior of organisms. From a practical standpoint, however, the most characteristic feature of any particular organism is the effect it has on the animal body, i. e., whether it produces harmful effects or not and the manner in which it does this. On this basis it would appear desirable to consider all of these organisms together as one species. The objection may, however, be raised as to the significance of the nontoxic culture No. 9 which was derived from a cell originally toxic. This culture has produced identical results with No. 7 throughout the cultural study, and still it differs in the essential property—that of producing toxin. The culture No. 7 itself became nontoxic before the study was completed, and there was evidence that some of the other cultures showed this same deterioration.

The solution of the problem in this case would appear to rest on the matter of stability of the properties considered. There was no evidence of any change in the proteolytic activity of any of the cultures studied. It is possible that nonproteolytic strains may arise from proteolytic or vice versa by a slow process of evolution, but there was no indication of such transformation. The toxin-producing property may, however, be lost in a short period of time.

On this basis the proteolytic activity of the cultures studied would seem to be the factor of most importance and would seem to justify the separation into two species. This division is supported by morphological evidence, as previously pointed out.

Since the Lister culture No. 94 corresponds so closely with the original van Ermengem strain, this strain should be considered the type species to be designated as *Clostridium botulinum* in accordance with the new genus designation.[5]

[5] The type of toxin produced by the strain originally isolated by van Ermengem is in question. It was surmised by the writer that the Lister Institute strain No. 95 labeled Madsen was the culture isolated by Madsen from mackerel, inasmuch as this is the only culture described in the literature by Madsen. The toxin produced by the mackerel culture was neutralized by the antitoxin of van Ermengem's strain (Kolle u. Wassermann). Information from Prof. Madsen, however, is to the effect that the Lister Institute culture is not the culture isolated from mackerel.

The fact that the van Ermengem culture was not highly toxic for chickens would indicate that it was not a type A culture, but rather a type B or C.

The type C organisms though differing in certain respects from this culture—such as failure usually to produce gas in solid culture media and in certain carbohydrate reactions—are so closely related to the Lister Institute culture that it seems desirable to group them with it.

Regarding the organisms of the type A group and the two type B cultures Nos. 5 and 19, these form a distinct group from a cultural point of view as well as in other respects.

They are strongly proteolytic, produce powerful odors, at least in certain media, and usually have a high thermal death point. They are actively motile, possessing numerous flagella and producing spores which in general swell the whole rod instead of appearing at once in the terminal position. These variations from the strains described would seem to justify a species designation differing from these and the name *Clostridium parabotulinum* is proposed for these.

"Type" as first used by Mrs. Burke refers to the kind of toxin produced, and the terms type A, type B, and type C, referring to toxin, should be retained, type A referring to cultures which produce the same kind of toxin as the Boise City strain, type B those which produce the same toxin as that produced by the Nevin strain, and type C those which produce a toxin which is neutralized by the antitoxin produced by inoculating the toxin of the Saunders strain or similar strains.

A definition of the term "proteolytic" in connection with the classification of these and other organisms is desirable. Miller and Reddish call attention to the indefinite meaning of this term as generally used. The question arises whether an organism liquefying gelatin should be considered "proteolytic" or not. "Proteolytic" as now used is a general term and these authors propose the use of specific terms to designate the kind of protein acted upon, as geletinolytic for organisms liquefying gelatin, sarcolytic for those digesting meat, ovolytic for those digesting egg albumen, etc. They propose that classification grouping be based on the action on a particular protein and suggest meat for this purpose, designating those organisms which digest meat as "sarcolytic" and those not digesting meat as "nonsarcolytic."

The writer concurs in the suggestion that a particular protein substance be used as a basis for classifying organisms in one or another group but believes that the use of coagulated hen egg albumen is a more satisfactory material for this purpose than meat, for the reason that it is a purer protein, and more uniform in composition than meat, which may be from different species of animals, or from different parts of the same animal, and therefore variable in composition. The results are also more definite and there is no question as to whether the egg white has been acted upon, if it is cut in pieces, preferably in cubes with straight edges. A slight action is indicated

by a wearing down of the edges and a tendency for the egg white to become less opaque, and to become brownish in color. The coagulated egg white in broth was a perfectly satisfactory medium in this work, and the differentiation between what has been designated as "proteolytic" and "nonproteolytic" was apparent to the extent that in the former the egg white had completely disappeared after an extended period of incubation, so that the medium presented the appearance of sterile broth with a slight flocculent precipitate at the bottom of the tube and the former showed the egg white in the same condition as the uninoculated control.

Using egg white therefore as a basis of differentiation between protelytic ("ovolytic") and nonproteolytic ("nonovolytic") it seems proper to designate such organisms of this group as produce a toxin which brings about symptoms of botulism in animals and which digest coagulated egg white as *Clostridium parabotulinum* and those which produce a toxin which acts in a similar way but which do not digest egg white as *Clostridium botulinum.*

The use of the term "parabotulinus" by Seddon for the organism described in connection with a study of bulbar paralysis of cattle does not seem justified in view of the fact that not sufficient cultural data are given. The description given allies it for the most part to the type C described in this paper.

II. FURTHER DATA ON TYPE C STRAINS.

Further data concerning the new type *C. botulinum* type C in addition to that already published have been accumulated in the course of this work.

THERMAL DEATH POINT.

Although type C has not been encountered in this country in any of the outbreaks of botulism thus far reported it can not be definitely stated that it has never been concerned in any human outbreak. It is not impossible that certain of the European outbreaks have been due to this type. The majority of the outbreaks in Europe have been caused by the consumption of such food substances as sausages and smoked meats, in which the matter of a high thermal death point of the organism is usually not of importance as is the case with those organisms found in canned goods, to which most of the outbreaks in this country have been ascribed. The thermal death point of van Ermengem's strain was 85° C. for one-fourth hour or 80° C. for one-half hour.

A preliminary test of the type C culture originally isolated indicated a thermal death point of a temperature of 93° to 95° C. maintained for a period between one-half and one hour. This is lower than that reported for certain of the strains concerned in the outbreaks of

botulism in this country, due to the eating of canned goods, but not for all. Five other strains have since been acquired and further data concerning these has been obtained showing that some strains are more resistent than the one first reported. The question naturally arises whether this type would be dangerous in canned goods.

A rough estimation of the thermal death points of the spores of all the type C strains and of the type B culture No. 21 (Lister Institute No. 94) was obtained by heating small amounts of 72-hour-old cultures containing spores at varying temperatures for 15 minutes, then planting the heated cultures in meat media, and incubating. The survival of spores was indicated by growth in the meat media as follows:

Growth of type C strains after heating for 15 minutes (48 hours' incubation).

Culture No.	85° C.	90° C.	95° C.	Boiling.
7 (type C)	+	+	+	+
11 (type C)	+	+	+	+
13 (type C)	+	+	+	+
15 (type C)	+	+	−	−
				+[1]
23 (type C)	+	+	+	+
25 (type C)	+	+	+	−
21 (type B)	+	−	−	−

[1] 72 hours.

This test showed that the type C strains are considerably more heat resistant than the nonproteolytic type B strain. The results obtained with the latter correspond with those obtained by van Ermengem.

Another test was then carried out at a boiling temperature for longer periods of time with spore suspensions. The cultures were grown in the cooked meat medium as before for a period of 72 hours. The fluid portion of the culture was filtered through sterile filter paper and heated to a temperature of 70° C. for 15 minutes to destroy vegetative forms. The reactions of the cultures were tested and found to vary between pH 6.2 and 6.4. The number of spores contained in each suspension was determined by making dilutions of each of the spore suspensions ranging from one-tenth to one millionth and planting 1 c. c. of each of these dilutions in meat medium. The various spore suspensions were then distributed in Wassermann tubes of hard glass and heated in an open water bath maintained at a boiling temperature, the periods of exposure varying from 5 minutes to 1½ hours. After the requisite amount of heating the contents of the various tubes were transferred to meat medium tubes and incubated. The results are indicated in the following table:

Thermal death point of type C strains.

+ indicates growth; — no growth.

Culture No.	Number of spores.	Period of heating (minutes).				
		5	15	30	60	90
7	Between 100,000 and 1,000,000.	+ (24 hour)...	+ (72 hour)...	—	—	—
9	.....do.....	.....do.....	+ (24 hour)...	—	—	—
11	.....do.....	.....do.....	.....do.....	—	—	—
13	.....do.....	.....do.....	.....do.....	+ (48 hour)...	+ (48 hour)...	—
15	.....do.....	.....do.....	.....do.....	+ (42 hour)...	—	—
23	.....do.....	.....do.....	.....do.....	.....do.....	—	—
25	1,000,000 or over.....	.....do.....	.....do.....	.....do.....	—	—

Tests were carried out to determine whether the cultures surviving at the highest temperatures were toxic, with the following results:

Toxicity of heated cultures.

Mouse No.	Amount of culture.	Culture No.	Period of heating.	Symptoms.	Death.
2678	0.2 c. c.....	7	15 minutes.....	None.....	Survived.
2679	0.2 c. c.....	9	.....do.....	.....do.....	Do.
2680	0.2 c. c.....	11	.....do.....	.....do.....	Do.
2681	0.2 c. c.....	13	30 minutes.....	1 hour.....	10½ hours.
2682	0.2 c. c.....	15	.....do.....	.....do.....	3½ hours.
2683	0.2 c. c.....	23	.....do.....	.....do.....	2½ hours.
2684	0.2 c. c.....	25	.....do.....	None.....	Survived.
2685	0.2 c. c.....	13	60 minutes.....	2½ hours.....	10½ hours.

The culture which was most resistant to heat was therefore No. 13 and spores of this culture produced toxin after surviving a period of 1 hour at the temperature of a boiling water bath.

A comparison with some of the results obtained in determining the thermal death points of type A and B strains, as reported in the literature, is of interest in this connection. Thom, Edmondson, and Giltner report that the Boise strain from asparagus survived heating to 100° C. for one hour, but failed to grow in subcultures from tubes heated for two hours. Burke (1919, *b*) studied 10 type A and B strains. Among four heated to 100° C. in a water bath, one survived 15 minutes, another 20 minutes, a third 30 minutes and the fourth 60 minutes. Weiss determined the thermal death point of 16 strains and found that four were destroyed at a temperature below 95° C. maintained for 10 minutes. These include three strains which were concerned in human outbreaks—his No. 3 isolated by Dickson from the crop of a chicken that died after eating home-canned string beans, the same beans having caused the death of one person; No. 4 isolated by Dickson from the gizzard of a chicken dying after eating home-canned corn, which had poisoned one person and about 50 chickens, and No. 7 isolated by Dickson from the crop or gizzard of a chicken which died after the eating of home-canned asparagus

which had caused the death of four persons. Of the remaining four strains which were isolated in connection with human outbreaks of botulism, one, No. 11 (Nevin strain) showed growth after 56 minutes exposure to 100° C. in a water bath, but not after 64 minutes; another, No. 16 (Canton strain) grew after 180 minutes exposure to 100° C. but not after 210 minutes; another, No. 15 (Boise strain) grew after 210 minutes exposure to 100° C. but not after 240 minutes; and a fourth, No. 6, grew after 270 minutes exposure but not after 300 minutes. It is to be noted that the three strains having comparatively low thermal death points were from home-canned goods, while the two type A strains Canton and Boise with very high thermal death points were from commercially canned goods.

Esty and Meyer determined the thermal death point of 78 type A strains, 30 type B strains, and 1 nontoxic strain. Their results are reported in terms of a temperature of 105° C. At this temperature the heat resistance varied from 3 to 80 minutes. Dickson, Burke, Beck, Johnston, and King showed that among 40 strains heated at 100° C., the survival times varied between 30 minutes and 6 hours. It has been pointed out in several of these investigations that the age of spores, the number of spores, the composition and hydrogen-ion concentration of the media and other factors are concerned in determining the thermal death point.

The results indicate a wide range in the heat resistance of *C. botulinum* and *C. parabotulinum* strains and the results reported by Weiss with the three organisms of comparatively low thermal death point in particular show that occasionally outbreaks of botulism have occurred with organisms which are not as heat resistant as these organisms are usually thought to be. The survival of spores of type C in canned goods, and the production of toxin is therefore possible, since the tests show that the thermal death point is higher than some of the type A strains, which have been concerned in human outbreaks.

TEMPERATURE AT WHICH TYPE C TOXIN IS DESTROYED.

A test was carried out on guinea pigs to determine the temperature at which a type C fluid toxin produced from strain No. 13 one month previously, was destroyed. This toxin had a minimal lethal dose of 0.0002 c. c. for guinea pigs. Amounts of 1 c. c. of toxin were placed in test tubes and heated to varying temperatures for periods of 1 minute, 15 minutes, and 30 minutes, after which inoculations were made into guinea pigs. The results are shown in the accompanying table:

Effect of temperature on fluid toxin type C, March 16, 1923.

No.	Weight.	Toxin.	Amount.	Symptoms.	Death.
			c. c.		
2213	245	Heated to 60° C. for 1 minute	1.0	4 hours	8 hours.
2214	255	Heated to 60° C. for 15 minutes	1.0	do	9 hours.
2215	280	Heated to 60° C. for 30 minutes	1.0	do	8 hours.
2216	245	Heated to 65° C. for 1 minute	1.0	do	9 hours.
2217	255	Heated to 65° C. for 15 minutes	1.0		10 hours.
2218	280	Heated to 65° C. for 30 minutes	1.0		9 hours.
2219	245	Heated to 70° C. for 1 minute	1.0		Do.
2220	250	Heated to 70° C. for 15 minutes	1.0		20 hours.
2221	280	Heated to 70° C. for 30 minutes	1.0	21 hours	28 hours.
2222	245	Heated to 75° C. for 1 minute	1.0	do	24 hours.
2223	250	Heated to 75° C. for 15 minutes	1.0	sl. 4 hours; def. 21 hours.	23 hours.
2224	275	Heated to 75° C. for 30 minutes	1.0	21 hours	12 days.
2225	245	Heated to 80° C. for 1 minute	1.0	do	44 hours.
2226	250	Heated to 80° C. for 15 minutes	1.0	sl. 21 hours; def. 28 hours.	68 hours.
2227	270	Heated to 80° C. for 30 minutes	1.0	None	Survived.
2228	245	Unheated	1.0	4 hours	9 hours.

The temperature at which the toxin of the particular strain used was destroyed is somewhat higher than that reported for the American type A and B strains. Thom, Edmondson, and Giltner found that the toxin of the Boise strain was destroyed almost immediately by heating to 75° C., or by heating 10 minutes at 73° C. Orr (1921) tested the toxins of 10 type A and B strains and found that they were comparatively thermolabile. At 80° C. they were all destroyed within from 30 seconds to 5 minutes, at 72° C. from 2 to 18 minutes, and at 65° C. within from 10 to 85 minutes.

The table shows that a temperature of 75° C. or less, maintained for not longer than 15 minutes, was not effective in destroying the toxin. The guinea pig inoculated with 1 c. c. of the toxin heated to 75° for 30 minutes showed definite symptoms and died in 12 days. A temperature of 80° C. for 30 minutes destroyed the toxin, the pig inoculated with this specimen showing no symptoms whatever. These results are comparable with those obtained by van Ermengem (1897, *a*) who found that a temperature of 80° C. maintained for one-half hour caused a deterioration to the extent that 10 c. c. injected into a rabbit produced only symptoms of cachexia and Landmann who found that the toxin of the Darmstadt strain was destroyed by a temperature of 75° C. maintained for 1 hour. In respect to the temperature destructive to the toxin the type C strain used appears therefore to be more closely related to the two strains just mentioned than to the American types A and B. It is of interest to note that the proteolytic strains having in general a high thermal death point produce toxins which are more thermolabile than those produced by the nonproteolytic strains with lower thermal death points.

DISTRIBUTION OF TYPE C CLOSTRIDIUM BOTULINUM.

As to the distribution of type C not much work has been done, and it can not at this time be stated that it is as common in the soil as the work of Meyer (1922) and his associates have shown types A and B to be, but indications are that it is widespread. The material used in this study was obtained from the following sections of the country: Missouri, Oklahoma, Illinois, and Maryland. Meyer and Dubovsky (1922, *a*) in examining soil from the various States in the Union apparently did not demonstrate definitely the presence of any strains which correspond to this type, though the statement is made that "it is naturally possible that a number of weakly toxic cultures are the result of the recently described anaërobe isolated by Bengtson." The medium used in their work was one which it is stated was intentionally made favorable for proteolytic anaërobes, and was a beef heart peptic digest liver broth, adjusted to a reaction of pH 7.4. The writer has not made a comparison of the medium used by Meyer and the cooked-meat medium used in the work here reported, but the reaction and the presence of liver in the medium would be favorable for the growth of the type C strains. Regarding the toxicity of the three types it has been shown that type C produces as strong a toxin as A or B, when conditions are favorable.

The investigation of the occurrence of type C in the soil in connection with this work was limited to examinations of the soil obtained from the localities in which the fly larvæ or other material was obtained for study and several other samples. The following illustrates the procedure carried out: Soil from the locality from which culture No. 11 was obtained was suspended in salt solution and heated to a temperature of 70–75° C. for one-half hour and planted in Kjeldahl flasks containing cooked-meat medium. Samples from four different parts of the yard in this case were used. Toxic cultures were obtained from three of these, as shown by intraperitoneal inoculation of mice with the growth obtained after 24 hours' incubation. Mice previously inoculated with the type C antitoxin were all protected, while those inoculated with a polyvalent antitoxin containing both A and B antitoxin died in about the same time as those not receiving antitoxin.

Three samples of soil collected by Dr. Robert Graham from two localities in which limberneck of chickens had occurred and one in which a horse had died with symptoms resembling botulism all showed the presence of type C cultures. Another sample of soil in the neighborhood of Great Falls, Md., where limberneck of chickens had been known to occur, also yielded type C cultures, and protection was afforded mice by type C antitoxin and not by either type A or B. Three other samples of soil obtained in the neighborhood of Washington were examined with negative or indefinite results.

In several instances subcutaneous inoculation of mice were made to test for the presence of tetanus spores and none were demonstrated. Among the eight soil samples tested, therefore, type C was demonstrated five times, and types A or B *C. parabotulinum* or *C. tetani* were not shown to be present.

As to the possible occurrence in other parts of the world, Meyer and Dubovsky (1922, *b*) isolated four cultures out of ten samples of soil from different parts of Holland which were toxic on feeding, but which were not neutralized by type A, B, or polyvalent antitoxins, which they consider may be similar to type C.

An organism recently described by Seddon which he associates with the disease known as bulbar paralysis in cattle occurring in Victoria, Australia, is very similar in its cultural characteristics, and may possibly be a representative of type C.

RELATIONSHIP TO ANIMAL DISEASES INCLUDING "LIMBERNECK" IN CHICKENS.

The relationship of this organism, as well as of types A and B, to certain animal diseases has not been investigated to the extent that is desirable, but it is not unlikely that all the members of this group are widely distributed and are concerned to some extent in certain such diseases. The fact that several manifestations of intoxication are brought about by the products of these organisms is recognized. Those animals which are highly susceptible die with very acute symptoms, but animals which are comparatively refractory or even susceptible animals receiving small doses of toxin show symptoms of emaciation, cachexia, and "unthrift." This has been frequently observed in guinea pigs in the course of this work. Such animals may die, but frequently recover. In this connection it may be stated, in reference to the original purpose for which this work was undertaken, namely, an investigation to determine whether any relationship existed between the toxic fly larvæ and poliomyelitis, that no symptoms of residual paralysis have been observed in any of the animals used. The same symptoms in animals have been observed throughout the work, whether type A, B, or C toxin was used.

As to the relationship of the organism to "limberneck" in chickens, data are given later in the paper to show that symptoms resembling limberneck which are sometimes followed by death are produced by the inoculation or feeding of the cultures or toxin to chickens. The development of these symptoms is not restricted to any particular type of *C. botulinum* or *C. parabotulinum*, but may be produced by type A, B, or C with the difference that much larger doses of B and of C than of A are required to produce the effects in chickens. The muscles of the legs and wings are usually affected

first, and this manifestation is indicative of less severe symptoms than is the case when the neck muscles are affected.

Regarding the relationship of the green fly *Lucilia cæsar* and other flies to the development of "limberneck" in chickens, the experiments of Wilkins and Dutcher; Saunders, Wisdom and White; Bishop and others have shown that under certain conditions chickens develop symptoms of "limberneck" when they consume fly larvæ or maggots. Ordinarily if the larvæ develop in the carcasses of animals which have not died of "limberneck" negative results have been obtained, indicating that the fly larvæ themselves are not harmful. If, on the other hand, the fly larvæ develop in the carcass of an animal which has died from limberneck, the chickens consuming these maggots or parts of the decayed carcass in time develop the disease. The difference in the results obtained is to be explained by the presence of the specific toxin-producing organism in the one case and its absence in the other. It is likely that carcasses left exposed do at times become spontaneously contaminated with the specific organisms in the soil and from these as a starting point sporadic outbreaks of "limberneck" in chickens may occur. That the species *Lucilia cæsar* is not alone concerned in the transmission of the disease is suggested by the isolation of the organism by the writer from the larvæ of *Lucilia serricata*. Bishop also states that *Chyrsomyia macellaria* reared in the carcasses of limberneck fowls and fed to healthy birds may produce limberneck. Saunders considers the domestic house fly *Musca domestica* not to be concerned in the transmission of the specific toxic factor.

The question as to the number of stages in the development of the fly through which the organism or toxin may be transmitted has not been answered definitely. It has been said that it may be carried "through the pupa and adult stage to the larvæ of the next generation reared in beef." The question arises as to how much of the transmission is actually through the various stages of the fly and how much is contamination mechanically transmitted through the food substance.

GROWTH OF TYPE C IN VARIOUS FOOD SUBSTANCES.

Several experiments were carried out to determine whether the type C strains develop in certain vegetable substances. The following specimens of canned goods were tested: Peas, asparagus, beets, olives, corn, string beans. Growth was not obtained in the asparagus, beets, olives, or string beans, but active growth as evidenced by gas production and odor was obtained in the peas and corn, both in tubes and in the cans. Samples from these fed to guinea pigs caused death in less than 19 hours, while pigs fed with the uninoculated food substance survived and pigs protected by type C anti-

toxin also survived. The spore suspensions were heated to 80° C. for one-half hour to destroy toxin. The sugar content of the corn and peas probably produced a more favorable medium than the other food substances used. The odors noted were of interest in view of the fact that in the laboratory culture media described the odor was very slight or not noticeable. The peas emitted a sour odor, while the corn had a very disagreeable odor which might better be described as fecal than otherwise. It is not certain that other spore-bearing organisms may not have already been present in the canned material which may account for the odors, and it is also possible that odors are produced in certain protein substances by type C cultures.

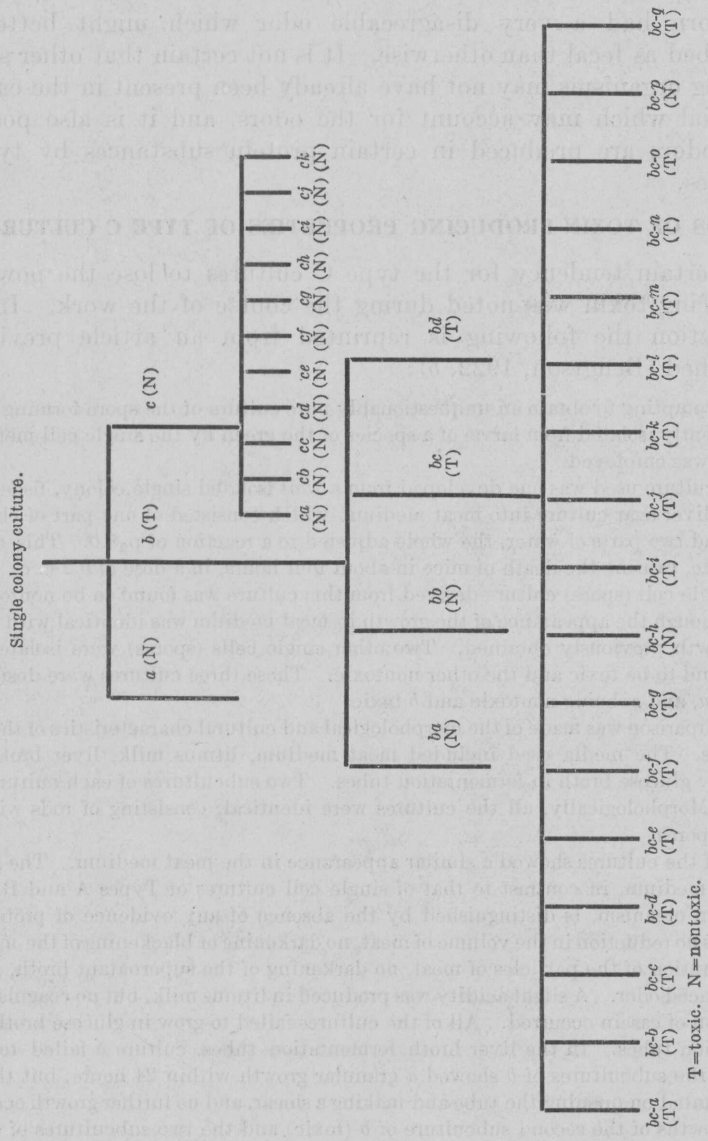
LOSS OF TOXIN-PRODUCING PROPERTIES OF TYPE C CULTURES.

A certain tendency for the type C cultures to lose the power of producing toxin was noted during the course of the work. In this connection the following is reprinted from an article previously published (Bengtson, 1922, *b*):

> In attempting to obtain an unquestionably pure culture of the spore-forming anaerobe recently isolated from larvæ of a species of the green fly the single cell method of Barber was employed.
>
> The culture used was one developed from a well isolated single colony, fished from a deep liver-agar culture into meat medium, which consisted of one part of chopped meat and two parts of water, the whole adjusted to a reaction of $p_H 8.0$. This culture was toxic, causing the death of mice in about four hours, in a dose of 0.2 c. c.
>
> A single cell (spore) culture derived from this culture was found to be nontoxic for mice, though the appearance of the growth in meat medium was identical with that of the growth previously obtained. Two other single cells (spores) were isolated; one was found to be toxic and the other nontoxic. These three cultures were designated *a*, *b*, *c*; *a*, and *c* being nontoxic and *b* toxic.
>
> A comparison was made of the morphological and cultural characteristics of the three cultures. The media used included meat medium, litmus milk, liver broth, and ordinary glucose broth in fermentation tubes. Two subcultures of each culture were used. Morphologically, all the cultures were identical, consisting of rods with terminal spores.
>
> All of the cultures showed a similar appearance in the meat medium. The growth in this medium, in contrast to that of single cell cultures of Types A and B of the botulism organism, is distinguished by the absence of any evidence of proteolysis. There is no reduction in the volume of meat, no darkening or blackening of the meat, no comminution of the particles of meat, no darkening of the supernatant broth, and no pronounced odor. A slight acidity was produced in litmus milk, but no coagulation or digestion of casein occurred. All of the cultures failed to grow in glucose broth fermentation tubes. In the liver broth fermentation tubes, culture *a* failed to grow. One of the subcultures of *b* showed a granular growth within 24 hours; but this was precipitated on opening the tube and making a smear, and no further growth occurred. The growths of the second subculture of *b* (toxic) and the two subcultures of *c* (nontoxic) were similar and unusual in that there was no evidence of growth in any of the tubes during the first 10 days. After this time a granular growth appeared. The cultures were left undisturbed and continued to grow, and all had produced gas to the amount of 20 to 25 per cent at the end of two weeks.

The similarity in morphology and in cultural reactions and the unusual behavior in the liver broth indicate the essential cultural identity of the toxic and nontoxic types.

Further single cell isolations were made from cultures *b* (toxic) and *c* (nontoxic). From *b*, two nontoxic cultures (*ba* and *bb*) and two toxic (*bc* and *bd*) were obtained. From *c*, eleven nontoxic cultures have thus far been obtained. From culture *bc*, 17 single cell isolations have been made, 15 of these being toxic and 2 nontoxic. The results are graphically presented in the accompanying diagram.



The two kinds of cultures have remained true to type, with one possible exception. Three months after the beginning of this work all single-cell cultures were retested on mice with the results given below, the subscript figure representing the number

of the transplant in meat media. Toxicity tests throughout have been made by inoculation of 0.2 c. c. of the culture intraperitoneally into mice. Characteristic symptoms usually develop within an hour or two and death occurs soon thereafter.

a_6(N)..... S.	c_5(N)..... S.
b_9(T)..... +3 hrs.	
ba_4(N)..... S.	bc_4(T)..... +2½ hrs.
bb_5(N)..... S.	bd_4(T)..... S. (bd_1, bd_2, bd_3, all toxic).
bc-a_4(T)..... +5 hrs.	bc-j_3(T)..... +3½ hrs.
bc-b_4(T)..... + 2hrs.	bc-k_3(T)..... +2 hrs.
bc-c_4(T)..... +2 hrs.	bc-l_3(T)..... +7 hrs.
bc-d_4(T)..... +7 hrs.	bc-m_2(T)..... +2 hrs.
bc-e_3(T)..... +3 hrs.	bc-n_2(T)..... +1 hr.
bc-f_3(T)..... +4½ hrs.	bc-o_2(T)..... +2 hrs.
bc-g_3(T)..... +3½ hrs.	bc-p_2(N)..... S.
bc-h_3(N)..... S.	bc-q_2(T)..... +2½ hrs.
bc-i_3(T)..... +3½ hrs.	
ca_3(N)..... S.	cd_3(N)..... S.
cb_3(N)..... S.	ce_3(N)..... S.
cc_3(N)..... S.	

S=survived. +=died (hrs.)

All of the cultures except one gave results the same as when first isolated. The culture *bd* originally toxic had become nontoxic, though tests with cultures incubated for a longer period showed a low toxicity, the death of mice occurring after a period of delay.

All cultures, including six more single-cell cultures isolated from the nontoxic culture *c*, were again tested on mice a month later, and in this case the results were consistent throughout with those obtained when the single-cell isolations were first made, the culture *bd* proving fatal to mice in seven hours.

The study is of interest in that it has been demonstrated that certain individuals in cultures originally toxic may apparently spontaneously lose their property of producing toxin. Just what factors are concerned in this phemomenon are problematical. A slight difference in the composition or reaction of the medium suggests itself as a possible explanation; but successive transplants of nontoxic cultures have always proved nontoxic, and it thus appears that once a culture loses its property of producing toxin it is not regained, regardless of any slight difference in the medium. The passage, by feeding of the cultures through fly larvæ (*Lucilia sericata*), failed to change the toxic properties of the two types, all toxic cultures remaining toxic and all the nontoxic cultures remaining nontoxic at the end of the experiment. The results suggest the possibility of the transformation of toxic cultures of other toxin-producing anaerobes, such as the organisms of tetanus and of botulism, types A and B, into nontoxic types.

The cultures in this series have been retained at cold-room temperature (15° C.) and several toxicity tests have been carried out to determine whether there had been any change in the toxin producing properties of any of the cultures. The test shown above was carried out June 9, 1922. The cultures were transplanted to

fresh cooked meat medium tubes and tested July 13 and August 31 of the same year. The culture bd_4 in the July test showed some toxin production, the mouse dying in about 21 hours. The culture *b* also showed a certain deterioration, as the toxin produced did not kill the mouse until the following day (about 21 hours). The remaining cultures all produced the same results as on the previous test. In the test of August 31, *bd* had become nontoxic and *b* again showed a decreased toxin production. The series of cultures was then placed in the cold room and allowed to remain until June 13, 1923, at which time transplants were made to fresh cooked meat medium and the 48-hour growth tested on mice as before.

The results were consistent with those previously obtained except that cultures *b* and *bd* both appeared to have completely lost the toxin producing property. All the remaining toxic cultures were still toxic and the nontoxic were still nontoxic. All the descendants of the single cell culture *bc* (*bc-a* to *bc-q*) have remained true to type throughout the tests which were made on this series of cultures, all being toxic except *bc-h* and *bc-p*. The culture *bc-c* which was used in the comparative cultural study (culture No. 7) and which was transplanted oftener and allowed to stand at room temperature at times did, however, lose its property of producing toxin. Culture No. 11 (117) also became nontoxic before the study was completed. There is some evidence that heating brought about the loss of the toxin producing properties. In carrying out tests to determine the thermal death point of the type C cultures it was noted that culture No. 13 heated to temperatures of 85°, 90°, and 95° C. for 15 minutes was still toxic, but after heating to a boiling temperature for 15 minutes no toxin was produced. Other strains subjected to the same conditions, however, did not show the loss of this property.

Culture *cf* (nontoxic), which is No. 9 in the cultural study, was transplanted June 26, July 11, August 30, 1922, and January 1, April 14, June 13, and June 16, 1923. All of these transplants were nontoxic.

The development of nontoxic from toxic cells may be considered in the nature of a bacterial mutation accepting the definition of de Vries to the effect that a mutant is a species or individual exhibiting a *sudden variation* in some well marked character as distinguished from a gradual variation in which the new character becomes fully developed only in the course of many generations. Jordan (1922), discussing variations and mutations, states that "there is now no doubt that true mutations occur among bacteria as among the higher forms of life." Certain strains of *B. coli* which acquired the property of fermenting saccharose and raffinose, were considered as fulfilling the requirements of bacterial mutation in that the change (*a*) appeared suddenly without inter-

mediate stages; (*b*) was irreversible over long periods; (*c*) comprised well marked changes in certain characteristics; (*d*) did not involve all the cells of the parent strain (Jordan, 1915). Dobell considers a bacterial mutation as a "permanent change—however small it may be—which takes place in a bacterium and is then transmitted to subsequent generations." He considers that the word mutation does not imply anything concerning the magnitude of the change, its suddenness, or the manner of its acquisition. Cole and Wright emphasize the fact that "the criterion of a mutation is that it must appear in a known pure line."

The appearance of the nontoxic cultures arising from single cells isolated from a toxic culture may in accordance with the conceptions of de Vries and Jordan of mutation be considered as a true bacterial mutation. The change may be considered sudden in that it takes place within a comparatively short time and it appears to be permanent. No definite evidence has been obtained in this study to indicate that a nontoxic culture has ever reverted to a toxic one. The slight fluctuation in the case of the culture *bd* may be explained on the basis that when the culture began to decrease in toxicity, it contained both toxin and nontoxin producing individuals and that one or the other type overgrew the other at various times and on this depended the presence or absence of toxin.

The question arises as to whether the cells in the toxic culture from which single cell isolations were made consisted of both the toxic and nontoxic variety or whether at this stage they all had the capacity of producing toxin, and the outcome as to toxin production in the case of the particular cells isolated was dependent on the conditions in the tube of media in which the cell was planted.

As to the effect of the composition of the medium de Kruif working with pure line strains was able to show that the peptone in the media he used, favored the process of mutation in the case of the rabbit septicemia organism. If the original culture were maintained in undiluted rabbit serum there was no development of the mutant, which exhibited a peculiar granular growth, produced a different kind of colony, had a different acid agglutination zone and had a lower virulence. If the culture were kept in media of high peptone concentration, mutations were rapid and reached a degree of 90% of the total number of organisms in 5 or 6 days. In the case of the toxic and nontoxic cultures under discussion there were no marked differences in media as usually tubes from the same batch of media were used and the differences must have been minute.

It would appear that the development of nontoxic individuals among toxic ones occurred spontaneously in the culture from which the cells were isolated and that if a nontoxic individual were fished it reproduced as nontoxic cells exclusively. The toxic cells fished

appeared to show a high degree of constancy when maintained under proper conditions, but under less favorable conditions evidently mutations continue to occur within the culture.

TYPE C ANTITOXIN.

The specificity of the type A and B antitoxins as regards the A and B toxin has been considered on page 26, and the lack of specific action against type C on page 30. The lack of specific action of the type C antitoxin against type A and B toxins is indicated in the following table:

Specificity of type C antitoxin as regards type A and B toxins.

Guinea pig.	Weight.	Toxin, type A.	Approx. M. L. D's.	Type C antitoxin.	Death.
	g.	*g.*		*c. c.*	
1504	250	0.0000026	1	0.08	88 hours.
1505	280	.0000052	2	.0896	Do.
1506	250	.000013	5	.08	34 hours.
1507	300	.000026	10	.096	Do.
1508	240	.0000026	1	(1)	Survived.
1509	250	.0000026	1	(1)	8 days 7 hours.
1510	250	.0000052	2	(1)	97 hours.
1511	300	.0000052	2	(1)	98 hours.
		Toxin, type B			
1512	250	0.00000057	1	0.08	40 hours.
1513	290	.00000114	2	.0928	Do.
1514	250	.00000285	5	.08	31 hours.
1515	300	.0000057	10	.096	21 hours.
1516	285	.00000057	1	(1)	5 days 20 hours.
1517	300	.00000057	1	(1)	74 hours.
1518	285	.00000114	2	(1)	24 hours.
1519	285	.00000114	2	(1)	66 hours.

[1] No antitoxin.

No protection against even 1 minimal lethal dose of A or B toxin was afforded by the amount of antitoxin used, 0.08 c. c. of which in another test was shown to protect against 100 minimal lethal doses of the type C toxin as follows:

Guinea pig.	Weight.	Type C toxin.	Approx. M. L. D.'s	Type C anti-toxin.	Death.
	g.	*g.*		*c. c.*	
1498	250	0.0002	100	.08	Survived.
1499	270	.000216	100	.0864	Do.
1500	250	.000002	1	.....	5 days 2 hours.
1500	280	.00000224	1	.....	95 hours.

The type C antitoxin was shown to fulfill one of the requirements of the definition of a true soluble toxin or exotoxin, i. e., that it must neutralize the toxin in multiple proportions:

Neutralization of toxin by antitoxin in multiple proportions.

Guinea pig.	Weight.	Toxin.	Approx. M. L. D.'s	Anti-toxin.	Symptoms.	Death.
	g.					
1487	280	0.00000224	1	0.000896	4 days 17 hours	Survived.
1489	250	.000004	2	.0016	do	Do.
1491	250	.00001	5	.004	None	Do.
1493	260	.0000208	10	.00832	do	Do.
1495	265	.000053	25	.0212	do	Do.
1497	265	.000106	50	.0424	do	Do.
1499	270	.000216	100	.0864	do	Do.
1500	250	.000002	1		41 hours	5 days 2 hours.
1501	280	.00000224	1		do	95 hours.
1502	250	.000004	2		do	88 hours.
1503	300	.0000048	2		do	79 hours.

Some tests were carried out to determine whether the antitoxin is transmitted to the offspring through the maternal ancestor. A female goat which was being immunized with the type C antitoxin, gave birth to two young, both females. The serums of the mother goat and of the two kids was tested two or three days later with the following results:

Transmission of immunity to offspring.

Guinea pig.	Weight.	Toxin.	Antitoxin.	Tested for per c. c.	Symptoms.	Death.
			Goat 295 (mother goat).			
	g.	*g.*	*c. c.*	*Unit.*		
2228	245	0.0002	0.2	0.5	None	Survived.
2229	245	.0002	.1	1	do	Do.
2230	250	.0002	.02	5	do	Do.
2231	255	.0002	.01	10	do	Do.
2232	270	.0002	.005	20	do	Do.
2233	275	.0002	.004	25	17 hours (sl)	6 days 2 hours.
			Kid 295L.			
2234	245	.0002	.2		None	Survived.
2235	245	.0002	.1		do	Do.
2236	255	.0002	.02		do	Do.
2237	260	.0002	.01		do	8 days 5 hours.
2238	270	.0002	.005		do	Survived.
2239	285	.0002	.004		do	Do.
			Kid 295S.			
2240	245	.0002	.2		do	Do.
2241	245	.0002	.1		17 hours	4 days 16 hours.
2242	255	.0002	.02			16 hours.
2243	260	.0002	.01		17 hours	22 hours.
2244	270	.0002	.005			16 hours.
2245	285	.0002	.004		17 hours	23 hours.

Normal goat serum.

Guinea pig.	Weight.	Toxin.	Approximate M.L.D.'s	Serum.	Symptoms.	Death.
	g.	*g.*		*c.c.*		
2169	240	0.000002	1	0.1	17 hours	29 hours.
2170	245	.000004	2	.1	21 hours	70 hours.

The results of this test show that the serum of one of the kids possessed as high (or possibly a higher) number of units of antitoxin as the mother goat (25 units), while the other kid showed less than 1 unit.

The kids were again bled about 6 weeks later and the following test was made:

Transmission of immunity to offspring.

Guinea pig.	Weight.	Toxin.	Antitoxin.	No. of units tested for per c. c.	Symptoms.	Death.
			Kid 295L.			
	g.	*g.*	*c. c.*			
2549	255	0.0002	0.1	1	None	Survived.
2550	260	.0002	.05	2	do	Do.
2551	295	.0002	.02	5	42 hours	53 hours.
2403	250	.0002	.01	10	17 hours	40 hours.
2404	255	.0002	.004	20	do	26 hours.
2405	265	.0002	.005	25	do	28 hours.
			Kid 295S.			
2406	245	.0002	1.0	.1	do	28 hours.
2407	250	.0002	.5	.2	do	40 hours.
2408	260	.0002	.2	.5	20 hours	31 hours.
2409	275	.0002	.1	1	17 hours	28 hours.

It is seen that in the case of kid 295L the potency of the serum fell from 25 units to 2 units, while in the case of kid 295S, the potency fell from 0.5 of a unit to less than 0.1. It is apparent therefore that the antitoxin content of the serum is lost fairly rapidly.

A comparison of the results obtained in this test with those obtained by Theobald Smith in determining the immunizing effects of diphtheria toxin on the mother and offspring, using guinea pigs, is of interest. Guinea pigs were treated with diphtheria toxin for periods varying from 2 months 13 days to 4 months 21 days, one animal being treated 7 times, two 6 times, and one 3 times. The total amount of toxin injected in each case was very small owing to the great sensitiveness of the guinea pig, varying from 0.0023 c. c. to 0.0098 c. c. One or two litters of each of these pigs was tested for immunity, the period between the last injection of the mother and the birth of the litter varying from 3 months 24 days to 7 months 7 days. The litters of three of the pigs showed no resistance, all the pigs of each litter apparently showing the same lack of resistance. The two litters of the fourth pig showed marked resistance. The results indicate a variation in the degree of immunity acquired by the mothers, the passive immunity of the offspring being employed as a measure of the active immunity of the mother.

In the tests referred to, which have been made with *C. botulinum* toxin on the offspring of a goat in the process of immunization, it has been shown that there is a great difference in the amount of passive immunity conferred on the different members of one litter. The results also indicate that passive immunity is probably lost much more rapidly in the case of animals protected against *C. botulinum* toxin than in those protected against diphtheria toxin.

III. PROPERTIES OF TOXIN AND ANTITOXIN OF ORGANISMS CONCERNED IN BOTULISM.

TOXIN.

EFFECT OF PHYSICAL AGENTS ON THE TOXINS IN THE FLUID AND PRECIPITATED STATE.

The information published by a number of investigators on the matter of the stability of fluid and precipitated toxins as regards physical agents is considerably at variance. The stability of the toxin is one of great importance in the standardization and testing of antitoxins, and the question arises as to whether the fluid toxin is sufficiently stable to allow of its use in this state for testing purposes, as is done in the testing of diphtheria antitoxin, or whether it is necessary to obtain the toxin in a form which is more surely stable, as is true of tetanus toxin.

The physical agents concerned in changes in the toxin are light, air, temperature, and moisture. In conjunction with these, the reaction of the toxin and the composition of the toxin with reference to the material derived from the medium in which the toxin is produced, i. e., the impurities contained in the toxin, are factors of a chemical nature which are also concerned in the deterioration of toxin.

It is not possible to separate entirely the effects produced by each one of these factors, and the deterioration is probably due to combinations of the various factors.

The consensus of opinion seems to be that fluid toxin exposed to light and air undergoes rapid deterioration, but if kept sealed in the dark deterioration is much less. Van Ermengem (1897) states that the filtrate from the ham from which he isolated his organism did not lose in toxicity for eight months (0.001 c. c. minimal lethal dose for rabbits) when kept sealed in the dark, while the toxin obtained in filtrates of pure cultures in media deteriorated noticeably in two or three months. Differences in the composition of the filtrate or of the reaction would probably account for this variation. If the toxin was evaporated rapidly at 37° C. in a dessicator, the residue maintained its toxicity for several months. If, on the contrary, the toxin was allowed to evaporate slowly during 8 or 10 days at room temperature and with a large surface exposure, the toxicity was lost. The dry toxin obtained from pure cultures was also not very stable.

Brieger and Kempner found dried toxin very stable, even though exposed to the air, if it was protected from light.

Thom, Edmonson, and Giltner found a filtrate of the Boise strain rather resistant to direct and indirect light. An exposure of 40 hours to direct sunlight was necessary to destroy the toxin.

Preliminary tests of the fluid toxin made by the writer indicated that the fluid toxin deteriorated to some extent. The knowledge

gained at the Hygienic Laboratory in regard to the stability of tetanus toxin in the dry state (Rosenau and Anderson) led to the use of precipitated toxin for carrying out the work of standardization. A number of fluid *C. parabotulinum* toxins were, however, retained in the dark in sealed containers at cold room temperature and tests made at intervals to determine the rate of deterioration. Tests have also been made in the course of the work which attest to the stability of the precipitated toxin. The deterioration of the fluid toxins (types A and B) will be considered first. The toxin was filled into tubes with a small surface exposure, sealed and stored at a temperature of about 15° C. The reaction varied from pH 6.9 to 8.3.

The results indicated in the following tables and diagram were obtained with a type B toxin made with the Nevin strain (strain 5 used in the cultural study). (Table IV–V, Diag. I.)

TABLE IV.—*Deterioration of fluid toxin type B 602 (July 16, 1920, toxin).*

MINIMAL LETHAL DOSE.

Date.	Guinea pig.	Weight.	Dose per 250 g.	Death.
		g.	*c. c.*	
1920.				
July 19	795	250	0.00002	Survived.
	796	260	.00005	52 hours.
July 22	816	250	.000064	Survived.
	817	250	.000085	59 hours.
Aug. 13	855	250	.000085	86 hours.
Oct. 13	930	280	.00017	6 days 17 hours.
	931	295	.00023	5 days 17 hours.
	932	300	.00032	88 hours.
Nov. 15	938	245	.00017	4 days 3 hours.
	939	260	.00023	88 hours.
	940	270	.00032	77 hours.
Nov. 20	950	255	.00017	4 days 17 hours.
	951	265	.00023	71 hours.
	952	295	.00032	89 hours.
Dec. 16	959	250	.00012	Survived.
	960	270	.00017	88 hours.
	961	290	.00023	60 hours.
	962	300	.00032	61 hours.
1921.				
Apr. 2	1,151	270	.00023	Survived.
	1,152	270	.00032	Do.
	1,153	295	.00045	94 hours.
1923.				
Mar. 17	1,999	245	.00045	Survived.
	2,000	265	.0009	Do.
	2,001	265	.0018	6 days 2 hours.
	2,002	275	.0036	77 hours.
	2,003	290	.0072	64 hours.

TABLE V.—*Deterioration of fluid toxin type B 602 (July 16, 1920, toxin).*

TEST DOSE (100 M. L. D.).

Date.	Guinea pig.	Weight.	Dose of toxin per 250 g.	Dose of antitoxin per 250 g.	Death.
1920.		*g.*	*g.*	*c. c.*	
July 22	822	250	0.0064	0.0008	Survived.
	823	250	.0085	.0008	4 days 17 hours.
Aug. 13	860	250	.0085	.0008	Survived.
	861	270	.012	.0008	Do.
	862	275	.017	.0008	7 days 3 hours.
	863	280	.023	.0008	28 hours.
Oct. 13	933	265	.017	.0008	Survived.
	934	280	.023	.0008	4 days 4 hours.
	935	290	.032	.0008	41 hours.
Nov. 15	943	240	.017	.0008	Survived.
	944	260	.023	.0008	Do.
	945	265	.032	.0008	51 hours.
Nov. 20	953	240	.017	.0008	Survived.
	954	250	.023	.0008	Do.
	955	260	.032	.0008	66 hours.
Dec. 16	963	250	.017	.0008	Survived.
	964	250	.023	.0008	Do.
	965	270	.032	.0008	77 hours.
1921.					
Jan. 17	1,014	250	.032	.0008	11 days.
	1,015	300	.032	.0008	Do.
	1,012	240	.039	.0008	88 hours.
	1,013	300	.039	.0008	94 hours.
Apr. 2	1,156	270	.032	.0008	Survived.
	1,157	295	.045	.0008	7 days 3 hours.
	1,158	300	.063	.0008	56 hours.
1923.					
Mar. 17	2,004	250	.045	.0008	Survived.
	2,005	265	.09	.0008	5 days 10 hours.
	2,006	275	.18	.0008	24 hours.
	2,007	275	.36	.0008	18 hours.
	2,008	290	.72	.0008	17 hours.

The results obtained with this toxin indicate a rather slow but continued deterioration. The minimal lethal dose increased during the three days between July 19 and July 22, 1920, from about 0.00005 to 0.000085 c. c. Counting from July 22, when the first test was made for the L+dose (=test dose, which is approximately 100 minimal lethal doses) the increase in the minimal lethal dose and the L+dose may be represented thus:

	0	8½ months.	33 months.
M. L. D.	0.00085 c. c. (59 hours)	0.00045 c. c. (94 hours)	0.0018 c. c. (6 days 2 hours).
L+dose	0.0085 c. c. (4 days 17 hours).	0.045 c. c. (7 days 3 hours)	0.09 c. c. (5 days 10 hours).

In 8½ months the minimal lethal dose and the test dose were about 5 times as large as they were at the beginning of the test, while in 33 months the test dose was about 10 times as large as at the beginning and the minimal lethal dose about 20 times as large.

The rate of deterioration, while considerably more rapid than that of diphtheria toxin, appears nevertheless not to be too great to exclude the use of fluid toxin for test purposes if proper controls are used. The results indicate that the change is most rapid at the beginning and that the toxin becomes more stable after a week or two; e. g., in 10 days the minimal lethal dose of a fluid toxin B 602

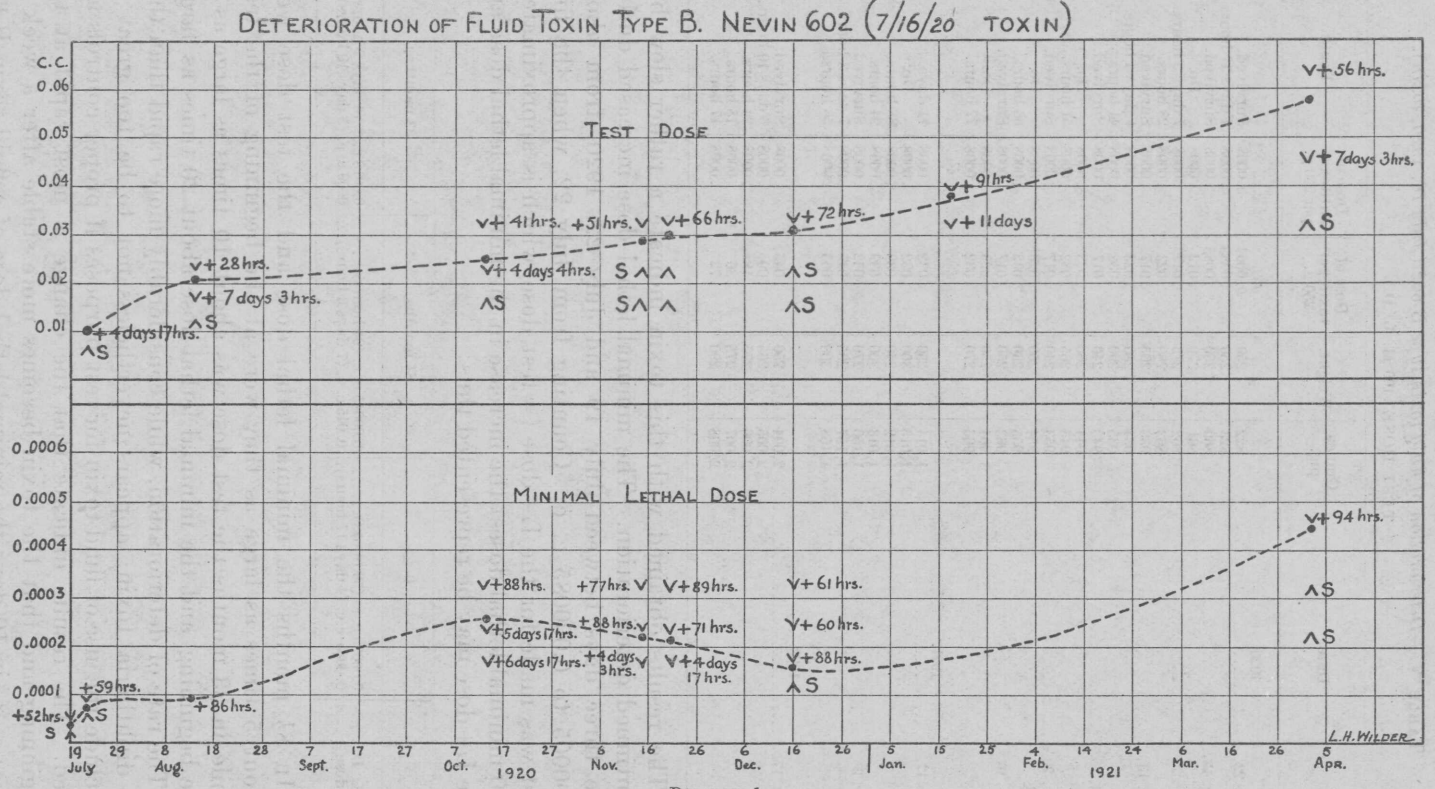

DETERIORATION OF FLUID TOXIN TYPE B. NEVIN 602 (7/16/20 TOXIN)
C.C.
0.06
0.05
0.04
0.03
0.02
0.01
TEST DOSE
V+ 56 hrs.
V+ 7 days 3 hrs.
V+ 91 hrs.
V+ 11 days
V+ 41 hrs.
+51 hrs.
V+ 66 hrs.
V+ 72 hrs.
V+ 28 hrs.
V+ 4 days 4 hrs.
V+ 7 days 3 hrs.
V+ 4 days 17 hrs.
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
MINIMAL LETHAL DOSE
V+ 94 hrs.
V+ 88 hrs.
+77 hrs.
V+ 89 hrs.
V+ 61 hrs.
±88 hrs.
V+ 71 hrs.
V+ 60 hrs.
V+ 5 days 17 hrs.
V+ 6 days 17 hrs.
+4 days 3 hrs.
V+ 4 days 17 hrs.
V+ 88 hrs.
+59 hrs.
+52 hrs.
+86 hrs.
L. H. WILDER
19 29 8 18 28 7 17 27 7 17 27 6 16 26 6 16 26 5 15 25 4 14 24 6 16 26 5
July Aug. Sept. Oct. 1920 Nov. Dec. Jan. Feb. 1921 Mar. Apr.

DIAGRAM 1.

(December 5, 1919) fell from 0.00003 c. c. to 0.0001 c. c.; in another, A 612 (December 6, 1919) fell from 0.0001 c. c. to 0.00016 c. c. in 5 days.

Several other fluid toxins which had been stored for periods of from one to three years were tested for deterioration as measured by increase in the minimal lethal dose. The results obtained with these may be summarized thus:

Deterioration of fluid toxins.

No. of toxin.	Date of first test.	pH.	Original M. L. D.	Time of death.	Date of last test.	pH.	M. L. D. at last test.	Time of death.
B 602	1919. Dec. 5	8.3	0.00003	+79 hours..	1923. Mar. 15 (39½ months).	7.6	0.016	+9 days 4 hours.
A 612	Dec. 6	8.2	0.0001	+59 hours..	.....do.....	7.8	0.008	+4 days 1 hour.
A 619, Boise strain	Dec. 5	7.8	0.0003	+4 days 14 hours.	1921. Jan. 14 (13½ months).	7.2	>0.001	
A, Canton strain...	Dec. 6	8.0	0.0003	+65 hours..	.....do.....	7.7	>0.001	
A, Lister Institute strain No. 95.	1922. May 21	.....	0.0005	+92 hours..	1923. Mar. 15 (10 months).	7.6	0.008	+5 days 18 hours.

The results obtained with the toxins B 602 and A 612 indicate that deterioration was greater than in the case of the B 602 toxin (July 16, 1920). The period of storage was longer and it is probable that the more alkaline reaction of the toxin was a factor in bringing about a greater deterioration.

The stability of the precipitated toxin is indicated by the results in determining the minimal lethal dose of a type A toxin which was retained *in vacuo* in the dark at a temperature of about 15° C. (Table VI).

TABLE VI.—*Stability of precipitated toxin.*

Date.	Guinea pig.	Weight.	Dose per 250 g.	Death.
1920.				
Mar. 5	275	255	0.000003	+92 hours.
Mar. 10	307	240	.000003	+96 hours.
Mar. 16	353	240	.000003	+67 hours.
	354	290	.000003	+6 days.
Mar. 20	389	250	.000003	+41 hours.
Mar. 29	422	320	.000003	+65 hours.
Apr. 20	481	330	.000003	+69 hours.
May 1	538	290	.000003	+4 days 12 hours.
	539	290	.000003	+84 hours.
	540	290	.000003	+78 hours.
May 6	550	250	.000003	+5 days 22 hours.
May 14	583	290	.000003	+82 hours.
May 20	608	295	.000003	+66 hours.
May 28	653	255	.000003	+82 hours.
June 18	732	250	.000003	+95 hours.
1921.				
July 22	1,380	250	.000003	+88 hours.
	1,381	270	.000003	Do.
Sept. 22	1,527	250	.000003	+62 hours.
	1,528	300	.000003	+4 days 6 hours
Sept. 29	1,563	260	.000003	+6 days 14 hours.
	1,564	280	.000003	+5 days 9 hours.
Oct. 18	1,635	280	.000003	
	1,636	270	.000003	+90 hours.
1923.				
Apr. 28	2,369	245	.000003	+4 days 17 hours.

Tests were also made to determine the effect of room temperature, incubator temperature, diffuse sunlight and direct sunlight on the same precipitated toxin (Tables VII–X).

TABLE VII.—*Effect of exposure of dry toxin at room temperature in the dark from March 29, 1920 (M. L. D. 0.000003 g.).*

MAY 6, 1920.

Guinea pig.	Weight.	Dose per 250 g.	Actual dose.	Death.
		[Exposed to room temperature 38 days.]		
	g.	*g.*	*g.*	
556	250	0.000003	0.000003	4 days 10 hours.
557	270	.0000045	.00000485	6 days 8 hours.
558	290	.0000068	.0000079	69 hours.
		[Controls.[1]]		
550	250	0.000003	0.000003	5 days 22 hours.
551	260	.0000045	.00000468	69 hours.
552	280	.0000068	.0000076	59 hours.

MAY 28, 1920.

Guinea pig.	Weight.	Dose per 250 g.	Actual dose.	Death.
		[Exposed to room temperature 60 days.]		
	g.	*g.*	*g.*	
662	315	0.000003	0.00000378	90 hours.
663	325	.0000045	.00000585	48 hours.
664	330	.0000068	.0000068	65 hours.
		[Controls.[1]]		
653	255	0.000003	0.00000306	82 hours.
654	265	.0000045	.00000477	22 hours.
655	275	.0000068	.0000075	54 hours.

TABLE VIII.—*Effect of room temperature and diffuse daylight on dried toxin (M. L. D. 0.000003 g.).*

MAY 6, 1920.

Guinea pig.	Weight.	Dose per 250 g.	Actual dose.	Death.
		[Exposed to room temperature and diffuse daylight 38 days.]		
	g.	*g.*	*g.*	
559	230	0.000003	0.000003	6 days 21 hours.
560	280	.0000045	.0000051	55 hours.
561	290	.0000068	.0000079	48 hours.
		[Controls.[1]]		
550	250	0.000003	0.000003	5 days 22 hours.
551	260	.0000045	.0000047	69 hours.
552	280	.0000068	.0000076	59 hours.

[1] The controls were pigs inoculated with toxin which was stored *in vacuo* in the dark at a temperature of 15° C.

TABLE VIII.—*Effect of room temperature and diffuse daylight on dried toxin (M. L. D. 0.000003 g.)*—Continued.

MAY 28, 1920.

Guinea pig.	Weight.	Dose per 250 g.	Actual dose.	Death.
[Exposed to room temperature and diffuse daylight 60 days.]				
	g.	*g.*	*g.*	
665	325	0.000003	0.0000037	76 hours.
666	330	.0000045	.0000059	72 hours.
667	390	.0000068	.000011	Do.
[Controls.[1]]				
653	255	0.000003	0.0000031	82 hours.
654	265	.0000045	.0000048	22 hours.
655	275	.0000068	.0000075	54 hours.

These results indicate that there was no deterioration of the toxin under the conditions of exposure for 60 days.

TABLE IX.—*Effect of a temperature of 37° C. on dried toxin (M. L. D. 0.000003 g.).*

APRIL 20, 1920.

Guinea pig.	Weight.	Dose per 250 g.	Actual dose.	Death.
[Exposed to 37° C. for 24 hours.]				
	g.	*g.*	*g.*	
484	340	0.000003	0.0000041	64 hours.
485	340	.0000045	.0000061	Do.
486	350	.0000068	.0000095	50 hours.
[Controls.[1]]				
481	330	0.000003	0.0000039	69 hours.
482	340	.0000045	.0000061	39 hours.
483	345	.0000068	.0000094	45 hours.

MAY 28, 1920.

Guinea pig.	Weight.	Dose per 250 g.	Actual dose.	Death.
[Exposed to 37° C. for 9 days.]				
	g.	*g.*	*g.*	
656	265	0.000003	0.0000022	4 days 19 hours.
657	295	.0000045	.0000053	89 hours.
658	300	.0000068	.0000082	19 hours.
[Controls.[1]]				
653	255	0.000003	0.0000031	82 hours.
654	265	.0000045	.0000048	22 hours.
655	275	.0000068	.0000075	54 hours.

[1] The controls were pigs inoculated with toxin which was stored *in vacuo* in the dark at a temperature of 15° C.

TABLE 9.—*Effect of a temperature of 37° C. on dried toxin (M. L. D. 0.000003 g.*—Continued.

JUNE 18, 1920.

Guinea pig.	Weight.	Dose per 250 g.	Actual dose.	Death.
[Exposed to 37° C. 24 days.]				
	g.	*g.*	*g.*	
735	250	0.000003	0.000003	65 hours.
736	270	.0000045	.0000049	64 hours.
737	285	.0000068	.0000078	53 hours.
[Controls.[1]]				
732	250	0.000003	0.000003	95 hours.
733	270	.0000045	.0000049	70 hours.
734	285	.0000068	.00000775	49 hours.

JULY 14, 1923.

Guinea pig.	Weight.	Dose per 250 g.	Actual dose.	Death.
[Exposed to 37° C. 110 days.]				
	g.	*g.*	*g.*	
2754	245	0.000003	0.00000298	Survived.
2755	245	.0000045	.0000044	Do.
2756	250	.0000068	.0000068	15 days 21 hours.
[Controls.[1]]				
2751	250	0.000003	0.000003	4 days 16 hours.
2752	255	.0000045	.0000046	Do.
2753	275	.0000068	.0000075	64 hours.

Table IX shows that the toxin was not affected by an exposure to a temperature of 37° C. for 24 days. After an exposure of 110 days at 37° the M. L. D. was increased to over 0.0000045 g., the guinea pig on 0.0000068 dying in 15 days.

The toxin was exposed to direct sunlight on an outside window-sill in a small, thin-walled agglutination tube and retained at a cold room temperature *in vacuo* during the intervals between the exposures. A slight amount of deterioration was evident after an exposure of 17 hours (Table X).

The results of the foregoing experiments attest to the stability of the dried toxin and indicate that it is comparable to precipitated tetanus toxin in this respect.

[1] The controls were pigs inoculated with toxin which was stored *in vacuo* in the dark at a temperature of 15° C.

TABLE X.—*Effect of direct sunlight on precipitated toxin (M. L. D. 0.000003 g.)*

MAY 6, 1920.

Guinea pig.	Weight.	Dose per 250 g.	Actual dose.	Death.
[Exposed to direct sunlight 1 hour.]				
	g.	*g.*	*g.*	
553	250	0.000003	0.000003	5 days 17 hours.
554	265	.0000045	.0000048	78 hours.
555	290	.0000068	.000007	48 hours.
[Controls.]				
550	250	0.000003	0.000003	5 days 22 hours.
551	260	.0000045	.0000047	69 hours.
552	280	.0000068	.0000076	59 hours.

MAY 28, 1920.

Guinea pig.	Weight	Dose per 250 g.	Actual dose.	Death.
[Exposed to sunlight 3 hours.]				
	g.	*g.*	*g.*	
659	310	0.000003	0.0000037	4 days 17 hours.
660	330	.0000045	.0000059	90 hours.
661	335	.0000068	.000009	57 hours.
[Controls.]				
653	255	0.000003	0.0000031	82 hours.
654	265	.0000045	.0000048	22 hours.
655	275	.0000068	.0000075	54 hours.

JUNE 18, 1920.

Guinea pig.	Weight.	Dose per 250 g.	Actual dose.	Death.
[Exposed to sunlight 6 hours.]				
	g.	*g.*	*g.*	
738	250	0.000003	0.000003	77 hours.
739	280	.0000045	.000005	35 hours.
740	290	.0000068	.000008	54 hours.
[Controls.]				
732	250	0.000003	0.000003	95 hours.
733	270	.0000045	.0000049	70 hours.
734	285	.0000068	.0000078	49 hours.

TABLE X.—*Effect of direct sunlight on precipitated toxin (M. L. D. 0.000003 g.)*—Con.

APR. 28, 1923.

Guinea pig.	Weight.	Dose per 250 g.	Actual dose.	Death.
		[Exposed to sunlight 17 hours.]		
	g.	*g.*	*g.*	
2362	245	0.000003	0.0000029	Survived.
2363	245	.0000045	.0000044	6 days 17 hours.
2364	265	.0000068	.0000072	4 days 17 hours.
2365	275	.000011	.000011	50 hours.
		[Controls.]		
2369	245	0.000003	0.000003	4 days 17 hours.
2370	250	.0000045	.0000045	75 hours.
2371	280	.0000068	.0000076	56 hours.

The stability of the toxin as regards the test dose or L+ dose is shown in the following table (Table XI):

TABLE XI.—*Stability of dried toxin stored in vacuo in the cold and dark.*

Guinea pig.	Weight.	Dose of toxin per 250 g.	Amount of antitoxin per 250 g.	Death.
1922.				
Dec. 28:	*g.*	*g.*	*g.*	
2353	240	0.00026	0.04	74 hours.
2354	265	.00026	.04	6 days, 3 hours.
1923.				
Jan. 3:				
2371	240	.00026	.04	55 hours.
2372	245	.00026	.04	44 hours.
Jan. 9:				
2389	245	.00026	.04	64 hours.
2390	250	.00026	.04	93 hours.
2391	255	.00026	.04	64 hours.
2392	290	.00026	.04	74 hours.
Jan. 30:				
2421	245	.00026	.04	59 hours.
2422	250	.00026	.04	Do.
2423	260	.00026	.04	64 hours.
Feb. 1:				
2437	250	.00026	.04	4 days, 4 hours.
2438	255	.00026	.04	72 hours.
2439	265	.00026	.04	50 hours.
2440	275	.00026	.04	88 hours.
Feb. 3:				
2460	250	.00026	.04	25 hours (symptoms not typical).
2461	250	.00026	.04	64 hours.
2462	250	.00026	.04	90 hours.
2463	255	.00026	.04	40 hours.
Feb. 8:				
2478	250	.00026	.04	55 hours.
2479	250	.00026	.04	83 hours.
2480	255	.00026	.04	47 hours.
2481	265	.00026	.04	55 hours.
Feb. 13:				
2510	250	.00026	.04	41 hours.
2511	255	.00026	.04	72 hours.
2512	260	.00026	.04	43 hours.
2513	270	.00026	.04	62 hours.
Feb. 20:				
2558	250	.00026	.04	5 days, 3 hours.
2559	255	.00026	.04	54 hours.
2560	260	.00026	.04	79 hours.
2561	270	.00026	.04	85 hours.

TABLE XI.—*Stability of dried toxin stored in vacuo in the cold and dark*—Continued.

G. P.	Weight.	Dose of toxin per 250 g.	Amount of antitoxin per 250 g.	Death.
1923.				
Mar. 6:	*g.*	*g.*	*g.*	
2582	250	0.00026	0.04	82 hours.
2583	250	.00026	.04	93 hours.
2584	255	.00026	.04	74 hours.
2585	265	.00026	.04	28 hours (symptoms not typical).
Mar. 13:				
2608	255	.00026	.04	71 hours.
2609	260	.00026	.04	Do.
2610	270	.00026	.04	36 hours.
2611	290	.00026	.04	64 hours.
Mar. 24:				
2620	245	.00026	.04	73 hours.
2621	250	.00026	.04	18 hours (symptoms not typical).
2622	275	.00026	.04	4 days, 17 hours.
2623	290	.00026	.04	Do.
Apr. 7:				
2657	250	.00026	.04	74 hours.
2658	250	.00026	.04	52 hours.
2659	255	.00026	.04	77 hours.
2660	260	.00026	.04	72 hours.
May 25:				
2667	245	.00026	.04	5 days, 22 hours.
2668	250	.00026	.04	76 hours.
2669	255	.00026	.04	64 hours.
2670	280	.00026	.04	96 hours.
June 9:				
2683	245	.00026	.04	64 hours.
2684	260	.00026	.04	5 days, 16 hours.
2685	270	.00026	.04	7 days, 19 hours.
2686	285	.00026	.04	64 hours.

EFFECTS OF CHEMICAL REAGENTS ON THE TOXINS.

A number of tests have been made to determine the effect of various chemical reagents on the toxin of *C. botulinum* and *C. parabotulinum*. Van Ermengem (1897) states that amyl alcohol, sulphuric ether, and benzol in acid or slightly alkaline media dissolve scarcely appreciable quantities of the toxin, and contact with them does not injure the toxin. The toxin is precipitated by tannin, lead acetate, zinc chloride, and neutral salts without losing its activity, while metallic salts such as the chlorides of gold, platinum, and mercury destroy it completely. The gases CO_2, H_2S and O exercised only a slight action on dissolved toxin.

Brieger and Kempner found the toxin was precipitated by alcohol, tannin and neutral salts. They also precipitated the toxin by treatment with zinc chloride and ammonium phosphate and found the purified toxin very labile. It was rapidly dissociated by ether, alcohol and oxydizing substances, but very resistant to reducing substances, such as sodium amalgam.

The effect of alcohol has been considered by several authors. Van Ermengem (1897) does not report any results with ethyl alcohol, but states that amyl alcohol does not injure the toxin. Schübel cites results obtained in treating toxin with alcohol showing that the toxin was weakened. In this country several investigators report

results obtained with ethyl alcohol. Armstrong, Story, and Scott report that if the toxin were diluted with alcohol up to 32 per cent subcutaneous or intraperitoneal inoculation of guinea pigs with amounts equivalent to 20 minimal lethal doses did not kill. Bronfenbrenner and Schlesinger (1921) found that 20–30 per cent dilutions of ethyl alcohol were capable of destroying large amounts of toxin *in vitro*, and the action was very rapid at 37° C. It was shown experimentally that alcohol administered by mouth to guinea pigs, immediately after a fatal dose of toxin had been given by mouth, prevented the death of the guinea pig, but if the alcohol were given subcutaneously no protection was afforded.

Hall and Davis used ethyl alcohol for detoxifying spores. The results obtained indicated that the alcohol reduced the toxicity to one-tenth of its original value.

Several investigations have been made to determine whether certain substances may be added to prevent the development of toxin in canned foods. Among these are salt, glucose, cane sugar and lemon juice. Wyant and Normington tested the effect of various percentages of salt on 19 strains of *C. parabotulinum*. All except two of the 19 strains, showed growth within 7 days in media containing 1 to 5 per cent of salt. The two strains were positive at the end of 27 days. Several strains grew in media containing 10 per cent of salt. It is probable that in this study only American strains were used. Van Ermengem (1897) states that the growth of his strain was inhibited completely by 6 per cent of salt. Thom, Edmondson, and Giltner found that the Boise strain grew and produced toxin in media containing 5 per cent of sodium chloride but not 8 per cent. It is likely that there is a certain variation as regards tolerance of sodium chloride among different strains. As pointed out by Wyant and Normington, the effect of reaction is also to be considered in connection with the inhibiting effect of salt.

The effect of glucose on the growth of the Boise strain was tested by Thom, Edmonsdon, and Giltner. Various percentages of glucose were added to tubes of meat medium and the cultures incubated for a week. There was growth and toxin production in tubes containing up to 35 per cent of glucose but none in tubes containing 45 per cent.

Dickson, Burke, and Ward determined the amount of cane sugar which was inhibitory to the development of the organism and its toxin. Percentages of cane sugar up to 64 per cent in beef broth media failed to prevent the formation of toxin. The toxin produced was however less strong than that produced in controls.

The effect of lemon juice on the growth of the spores and the development of toxin was also tested by Dickson, Burke, and Ward. It was found that while lemon juice in the proportion of 5 to 7 per cent

did not prevent the growth of the organisms, the thermal death point of the spores was lowered markedly when they were heated in an acid medium with this concentration of lemon juice. No toxin was produced in string beans and green peas contaminated with spores and containing these percentages of lemon juice and 2 per cent of salt when heated in boiling water for 60 to 90 minutes, while controls without the lemon juice contained numerous spores and a highly potent toxin.

Burke, Elder, and Pischel tested a large number of substances to determine whether they would be useful for the nonspecific treatment of botulism. These include olive oil, liquid petrolatum, butter, milk, egg white, egg yolk, sugar, brain, gelatin, Gram's iodin solution, liquid soap, and other substances. For the most part the effects were slight or negative. Tests were usually made by mixing a definite amount of toxic filtrate (2 to 8 minimal lethal doses) with the substances being tested and injecting into guinea pigs, or by feeding rabbits, the substance used for treatment being administered immediately after the toxin or at certain later intervals. Several c. c. of olive oil emulsified and mixed with 3 minimal lethal doses prevented the death of guinea pigs. When 4 minimal lethal doses of toxin were used, however, the pigs died. The protective action seemed to be due to a slowing of the absorptive process. The death of rabbits fed with toxin could be delayed by feeding olive oil immediately afterwards and at intervals thereafter. Somewhat similar results were obtained with liquid petrolatum. A very slight retardation was obtained when butter was used. Kempner and Schepilewsky had previously shown that emulsified oil would protect mice against 2 minimal lethal doses of toxin.

Iodin to which starch had been added to prevent tissue necrosis (due to the effect of the iodin) was found by Burke, Elder, and Pischel to afford some protection against the toxin when injected with the toxin, but not when injected separately. Gram's iodin solution afforded some protection also when fed to rabbits immediately after the toxin. Sodium hydroxide, potassium permanganate, and liquid soap had some neutralizing action on the toxin when they were mixed with the toxin, incubated and injected subcutaneously into guinea pigs.

It was found that 3 c. c. of apple vinegar protected against 4 minimal lethal doses of toxin when the two were mixed *in vitro* and injected subcutaneously into guinea pigs. The protective action was ascribed to the alcohol contained in the vinegar, since acetic acid and sodium acetate do not neutralize the toxin. Vinegar force fed to a rabbit after the toxin also seemed to afford some protection.

Effect of Acids and Alkalies on the Toxins.

In contrast with tetanus toxin which is unstable in the presence of acids, particularly mineral acids, the toxins of *C. parabotulinum* and *C. botulinum* are very resistant to acids. Kitasato found that HCl in the proportion of 0.55 per cent caused a deterioration of tetanus toxin within 1 hour, while an amount of 0.1365 per cent completely destroyed it in 24 hours. Fermi and Pernossi determined that tetanus toxin was destroyed by the gastric juice through the activity of the hydrochloric acid and not by the pepsin.

Van Ermengem states in regard to the toxin of *C. botulinum* that tartaric acid and lactic acid in the proportion of 1 to 3 per cent and HCl in the proportion of 0.5 to 1 per cent did not lower the toxicity of the filtrate after retaining 24 to 36 hours at 35°. Sodium carbonate, on the other hand, in the proportion of 0.5, 1, and 3 per cent, and ammonia in the proportion of 0.5 and 1 per cent when allowed to act on the toxin for 24 to 48 hours rendered it innocuous to rabbits, symptoms not being produced when injections with 50 to 100 times the fatal amount of untreated toxin were made. A solution of pure toxin in water was rendered instantly inert when an equal volume of 3 per cent sodium carbonate was added. It was not possible to restore the toxicity by neutralization with acid.

Other authors have confirmed the observations of van Ermengem (1897). Landmann found that 20 per cent of a normal alkali solution destroyed the toxin of the Darmstadt strain in a short time, while the same amount of normal acid produced no effect. Schübel found that when the concentration of acid was increased beyond a certain point the toxin deteriorated or was destroyed.

Bronfenbrenner and Schlesinger (1922, *a*) in testing the effect of acids and alkalies on botulinus toxin found that the strength of the toxin was decreased about 10 times when kept for 24 hours at a reaction of pH 8.0 When adjusted to the same acidity as that which occurs in the stomach pH 4.0, on the other hand it was noted there was not only no deterioration of the toxin but an actual increase so that the toxin became many fold more toxic than previously. The enormous increase reported, from a minimal lethal dose of 3×10^{-6} c. c. for a mouse to 3×10^{-18} and occasionally 3×10^{-21} has not been confirmed. They found that no larger amount of antitoxin was required to neutralize the highly potent acidified toxin than the original toxin; i. e., the combining power of the toxin was not increased with the increase in toxicity. Filtrates of young cultures were more likely to yield such high potency toxins on acidification.

Tests made by the writer indicate a considerably increased toxicity as the result of acidification to a reaction of pH 4.0 with HCl but not to the extent reported by Bronfenbrenner.

In one test a toxin produced by growing a type A culture (Memphis) for 48 hours in the meat medium of reaction pH 8.5 and containing 1 per cent of glucose was used. The M. L. D. of the toxin as shown by results in the control mice was 0.00032 c. c. With the toxin adjusted to pH 4.0 the mouse inoculated with 0.000064 c. c. died, while the mouse inoculated with 0.0000128 c. c. showed definite symptoms. The strength of the toxin was therefore increased at least fivefold. Practically the same results were obtained with 3-day, 4-day, and 5-day old toxins of the same batch. The mice on the 0.0000128 c. c. dose of the 4-day and 5-day toxins died with doubtful symptoms.

In another test a 72-hour filtrate of the Memphis type A strain of a reaction of pH 7.0 at the time of filtration was adjusted to pH 4.0 and tests made by intraperitoneal inoculation of mice. This toxin was a weaker toxin than the one previously used. The results obtained are shown in the following table.

Mouse No.	Dose.	Adjusted toxin.		Mouse No.	Unadjusted toxin.		
		Symptoms.	Death.		Dose.	Symptoms.	Death.
	c. c.				c. c.		
2482....	$1/10^{2}$	1 hour.....	3 hours.	2487....	$1/10^{2}$	3 hours.....	11 hours.
2483....	$1/10^{2.5}$	2 hours.....	4½ hours.	2488....	$1/10^{2.5}$	4½ hours.....	28 hours.
2484....	$1/10^{3}$	3 hours.....	6 hours.	2489....	$1/10^{3}$	None.....	Survived.
2485....	$1/10^{3.5}$	4 hours.....	28 hours.	2490....	$1/10^{3.5}$	.....do.....	Do.
2486....	$1/10^{4}$	5 hours?..... 28 hours def.....	31 hours.	2491....	$1/10^{4}$	.....do.....	Do.
2500....	$1/10^{4.5}$	46 hours.....	48 hours.				
2501....	$1/10^{5}$	(?).....	45 hours.				
2502....	$1/10^{5.5}$	None.....	Survived.				
2503....	$1/10^{6}$	.....do.....	Do.				

The increase in the toxicity in this test was from $1/10^{2.5}$ to $1/10^{4}$, considering mice Nos. 2486 and 2488. This represents an increase of toxicity from 1/316 c. c. to 1/10000 c. c. which is approximately 32 fold. Considering mice Nos. 2500 and 2501 the increase would be 100 to 320 fold.

The results therefore show a definite increase in toxicity and it is possible that with favorable conditions the toxicity might be increased still more, but further experimental work is necessary to show this.

Factors such as the particular strain used, the culture medium, the time and temperature of incubation of the cultures, and the length of time the acid is allowed to act on the toxin are factors to be considered.

The results of Bronfenbrenner in regard to the stability of the precipitated toxin in acid solution was confirmed. There was no increase in potency when a precipitated type B toxin was acidified with HCl to the reaction of pH 4.0.

FOOD SUBSTANCES IN WHICH THE TOXINS HAVE BEEN ELABORATED.

Food substances have been found suitable media for the development of *C. parabotulinum* as evidenced by the demonstration of the organism or the toxin in the food substances as enumerated below. The food substances in question have either been concerned in outbreaks of botulism or it has been experimentally shown that the organism would grow and develop its toxin in them. Before the organism of botulism was isolated by van Ermengem, various food substances, principally meat and fish products, were implicated in reported outbreaks of botulism. These are adequately covered in the monograph of Dickson (1918, *a*) on botulism. A number of outbreaks of botulism have occurred in this country in which various food substances have been assigned as the causative factors on epidemiological grounds. These are referred to in the publication of Geiger, Dickson, and Meyer on the epidemiology of botulism and in the monograph of Dickson (1918, *a*).

The number of food substances from which the organism has actually been isolated, as reported in the literature, include the following: Ham (van Ermengem, 1897, 1912; Römer); mackerel (Madsen, 1912); preserved white beans (Landmann), ripe olives (Armstrong, Story, and Scott; Jennings, Haas, and Jennings; Emerson and Collins; Sisco; Fontaine; Geiger, Dickson, and Meyer; De Bord, Edmondson, and Thom); olive relish (De Bord, Edmondson, and Thom); olives stuffed with pimento (De Bord, Edmondson, and Thom); spinach (Colver; Geiger, Dickson and Meyer; Koser); home-canned corn (Geiger, Dickson, and Meyer); string beans (Geiger, Dickson, and Meyer); asparagus (Thom, Edmondson, and Giltner); cottage cheese (Nevin); wild duck paste (Leighton); canned salmon (Morel and Martin). Among these type B has been reported 1 time (cottage cheese), and type A 11 times (ripe olives 5 times), olives stuffed with pimento 1 time, spinach 2 times, asparagus 1 time, string beans 1 time, wild duck paste 1 time. The type was not known or designated in 7 cases. In addition to these Randall reports the organism isolated from blood sausage and ham in two different outbreaks, but the evidence is not conclusive to prove that the organism was isolated in pure culture, though the symptoms as described indicate botulism.

In certain outbreaks the organism has been isolated from the crop of chickens which have eaten of the same food as that presumably concerned in the outbreak. In this series of indirect isolations no foods are involved which are not included among those listed above except apricots (home-canned) (Dickson, 1918, *b*), and possibly home-canned white beans or home-canned pickled mackerel and herring (Geiger, Dickson, and Meyer); type A was isolated in both cases.

In some outbreaks the evidence of intoxication by *C. botulinum* has been adduced by isolation of the organism from the stools of the patients. In these outbreaks no foods have been involved as reported in the literature which are not included among those named. For an account of these outbreaks, reference may be made to the epidemiological study of Geiger, Dickson, and Meyer.

In an outbreak ascribed to canned beets (Randall) a type B organism was isolated from the jejunum of one of the victims.

Dickson (1918, *a*) showed experimentally that the toxin may be produced in string beans, green peas, green corn, asparagus, artichokes, peaches, apricots, crushed apricot stones; Dickson, Burke, and Ward report that pears and prunes may also form suitable culture media for the development of the toxin. The use of various media in the laboratory shows that the organism grows in beef, pork, mutton, in brain and liver and in milk. Edmondson, Giltner, and Thom found that toxin was produced in sterilized milk but not in pasteurized milk from a city dairy. In milk pasteurized in the laboratory and held at 37° C. toxin was produced after 3 days. Toxin was produced in laboratory pasteurized milk in 2 days at a temperature of 30° C. but none in milk retained at a temperature of 20° C. Thom, Edmondson, and Giltner produced the toxin in Swiss cheese.

EFFECT OF TOXIN ON THE BODY.

Susceptibility of Various Animals.

The elaboration of these different "types" of toxin which are absolutely specific as to neutralization by their respective antitoxins, and which in general are similar in their action on animals is of interest. Certain differences as to quantitative and perhaps qualitative effect on different species of animals, however, is evidence of the complexity of the reaction which takes place between the toxin molecule and the substance of the nerve tissue.

Van Ermengem (1897) studied at considerable length the action of the toxin produced by the Ellezelles strain first isolated by him. He reports the effects produced by subcutaneous, intraperitoneal, intravenous, intracraneal, intraocular inoculations, and by feeding. Rabbits, guinea pigs, mice were most susceptible. 0.0003–0.001 c. c. of a culture injected subcutaneously was fatal for rabbits, 0.0001–0.00005 c. c. for guinea pigs, and the susceptibility of mice was extremely high. 0.01 to 0.001 c. c. produced symptoms and death in small rhesus monkeys. Goats and horses were very susceptible, 0.03 c. c. being a fatal dose for a goat. Amounts of 0.1–1 c. c. of toxin produced characteristic symptoms and death of cats in 6 to 8 days. White rats were reported as slightly susceptible, while dogs and chickens could withstand enormous doses, in the case of dogs 10 to 30 c. c. causing only emaciation and the formation of an abscess. Pigeons

succumbed to injections of 0.1 to 0.5 c. c.; frogs and fishes were found completely refractory.

Monkeys, guinea pigs, and mice were found most susceptible to intoxication by ingestion. Rabbits, on the other hand, which are very susceptible when inoculated, were found to be resistant to large doses of toxin (5–10 c. c.) by mouth. Cats would also withstand very large doses when fed, and the dog, rat, and hen were also resistant to enormous doses by this route.

Schubel reports that frogs and fishes were susceptible. Fishes were affected both when immersed in toxic fluid and when injected. He also made tests on lower forms of life and found that copepoda, earthworms, tadpoles, and snails were susceptible. He reaches the conclusion that with the development and differentiation of a nervous system there is a corresponding increase in the susceptibility. The source of the strain which this investigator used is not stated, and it is therefore not certain whether it was of the same or of a different type than van Ermengem's. Van Ermengem reported that the Ellezelles strain had no effect on frogs and fishes.

A number of tests as to the susceptibility of animals toward the types A and B American strains have been made. Thom, Edmondson, and Giltner report the following results with the Boise City strain. A dose of 5 c. c. of a toxin with a minimal lethal dose of 0.001 c. c. for guinea pigs (5,000 M.L.D.) was fatal to a 5-kg. dog when injected subcutaneously. Feeding of 13,000 M. L. D. at one time and 200,000 M. L. D. at another was not fatal to a dog of 20-kg. weight. Cows injected subcutaneously with 8,000 M. L. D. survived, while those injected with 200,000 M. L. D. died. Feeding of 7,000–8,000 M. L. D. did not affect cows. A hog injected with 50,000 M. L. D. died in 7 days, but feeding with 6,000 had no effect. Chickens fed with 10,000 and 40,000 M. L. D. died in 46 and 18 hours, but those fed with 1,000 and 5,000 M. L. D. survived.

Buckley and Shippen carried out tests with the Nevin (type B) strain and found that 10 c. c. of a 4-day culture poured on oat hay was fatal to a 2-year-old colt on the third day, while 0.2 c. c. of a 3-day-old culture poured on bran caused the death of a donkey in 6 days.

Seddon reports that the organism which he studied, and which possibly corresponds to the type C described in this paper, was toxic for rabbits, cattle, horses, and sheep, but much more toxic for horses than for cattle. Hart and Hayes found that about 300 minimal lethal doses of a type A toxin for a guinea pig was a fatal dose for a horse when inoculated. The results show therefore that horses are much more susceptible to this toxin than guinea pigs in proportion to weight. Cattle were more resistant; a young heifer withstood 2,000 minimal lethal doses of toxin when injected subcutaneously. A dose of 20,000 minimal lethal doses was, however, a fatal dose. Dickson

found that dogs could be killed by inoculating 1 c. c. of filtrate of type A cultures.

The susceptibility of chickens to the toxin has been the subject of considerable discussion. Dickson (1918, a) called attention to the discrepancy in the results obtained by van Ermengem and his own. Dickson found chickens to be "highly susceptible to subcutaneous injection and to feeding." It was noted that in a number of outbreaks which he investigated that chickens died from "limberneck" a disease in which "the clinical picture is entirely analogous to that which occurs in susceptible animals."

Buckley and Shippen state that chickens and dogs were refractory to cultures of the Nevin strain when fed with quantities as high as 30 c. c. daily for 3 to 4 days.

Graham and Schwarze propose a rapid differentiation of type A and B toxins in foods by feeding 1 to 5 c. c. of suspected material to chickens. Experimental work carried out by them, with unfiltered cultures of both types suggest to them that chickens are highly susceptible to type A toxin and refractory to type B. The same results they state are indicated by the bacteriologic investigation of sporadic outbreaks of "limberneck" in chickens in which only type A has been found.

Hart and Hayes tested the effect of type B filtrate on chickens and were able to produce symptoms of "limberneck" followed by death, by inoculating 15,000 M. L. D. subcutaneously; with type A filtrates 400 M. L. D. was a sufficiently large dose to cause symptoms of "limberneck" and death in chickens.

The following results were obtained by the writer in tests made on a quantative basis to determine the comparative susceptibility of chickens to the three types of toxins.

Susceptibility of chickens to different types of toxin:

Type A toxin (fluid), M. L. D. 0.0001 c. c.

SUBCUTANEOUS INOCULATION.

Chicken.	Weight.	Toxin.	Approximate M. L. D.'s for guinea pig.	Symptoms.	Death.
	g.	*c. c.*			
2384	1,300	0.02	200		11 days 18 hours.
2385	1,400	.2	2,000	24 hours	44 hours.
2386	1,500	2	20,000	19 hours	25 hours.
2387	1,600	1.0	100,000		18 hours.
2388	1,700	20	200,000		Do.
FEEDING.					
2389	1,400	0.02	200	None	Survived.
2390	1,500	.2	2,000	do	Do.
2391	1,500	2.0	20,000	19 hours	24 hours.
2392	1,600	10.0	100,000	do	76 hours.
2393	1,000	20.0	200,000	do	26 hours.

A precipitated toxin (M. L. D. 0.000003 g.) was also inoculated into two chickens.

Chicken.	Weight.	Toxin.	Approximate M. L. D.'s for guinea pig.	Symptoms.	Death.
	g.	*c. c.*			
2394	1,400	0.00069	200	48 hours	52 hours.
2395	1,500	.006	2,000	.....do	76 hours.

Type B toxin (precipitated), M. L. D. 0.0000004 g.)

SUBCUTANEOUS INOCULATION.

Chicken.	Weight.	Toxin.	Approximate M. L. D.'s for guinea pig.	Symptoms.	Death.
1235	725	0.04 c. c. (1/1000 dil.)	100	None	Survived.
1236	650	0.4 c. c. (1/1000 dil.)	1,000	.....do	Do.
1237	610	4.0 c. c. (1/1000 dil.)	10,000	44 hours	+4 days 21 hours.

FEEDING.

1232	645	0.4 c. c. (1/1000 dil.)	1,000	None	Survived.
1233	820	4.0 c. c. (1/1000 dil.)	10,000	.....do	Do.
1234	690	40.0 c. c. (1/1000 dil.)	100,000	48 hours	Recovered.

Chicken 1234 showed rather severe symptoms but recovered after about 12 days.

Type C toxin (precipitate) M. L. D. 0.000002 g.

SUBCUTANEOUS INOCULATION.

Chicken.	Weight.	Toxin.	Approximate M. L. D.'s for guinea pig.	Symptoms.	Death.
1183	760	0.5 c. c. (1/10000 dil.)	25	None	Survived.
1184	810	0.5 c. c. (1/1000 dil.)	250	.....do	Do.
1185	865	0.5 c. c. (1/100 dil.)	2,500	.....do	Do.
1186	980	50.0 c. c. (1/100 dil.)	25,000	42 hours	+4 days 12 hours.

FEEDING.

1181	790	0.5 c. c. (1/1000 dil.)	250	None	Survived.
1188	845	0.5 c. c. (1/100 dil.)	2,500	.....do	Do.
1189	905	5.0 c. c. (1/100 dil.)	25,000	.....do	Do.
1190	1,180	50.0 c. c. (1/100 dil.)	250,000	22 hours	Recovered.

Chicken 1190 developed severe symptoms becoming decumbent with wings and tail dropping but recovered in about six days.

The results indicate that the type A toxin is considerably more toxic for chickens than either type B or type C. The fatal doses in the inoculation tests are represented roughly by 2,000 guinea pig M. L. D.'s for type A, 10,000 for type B, and 25,000 for type C, which is in the proportion 1:5:12.5. If the chicken No. 2384 be considered which died later the ratio is 1:50:125. An average of these figures would probably be a good estimate of the difference in potency of the three toxins, and it may therefore be stated that the A toxin is roughly 25 times as toxic for chickens as the B and C toxins.

In the feeding tests about the same relationship holds good, the fatal dose in the case of type A being 20,000 guinea pig M. L. D.'s, while in the case of B and C 100,000 to 250,000 M. L. D.'s produced severe symptoms in chickens, but they eventually recovered.

The fatal feeding dose in all the tests is somewhat more than 10 times the fatal dose by inoculation.

Regarding the development of the disease "limberneck" in chickens under natural conditions it may be said that the amount of toxic material ingested is only limited by the capacity of the crop, and it is therefore easily possible that sufficient toxic material may be ingested to produce the disease with both types B and C. Fly larvæ to the number of 1,200 were removed from the crop of one of the specimens from which the type C organism was isolated. The largest amount of fluid fed in the experiments cited was 50 c. c. The toxin in carrion material or fly larvæ is probably in a much more concentrated form. In one test 25 c. c. of a culture of the larvæ from specimen No. 117 in the cooked meat medium caused death of a 1,200-gram chicken when fed, typical symptoms developing in 70 hours; while the meat from two tubes planted with the larvæ (approximately 5 to 6 grams) caused severe symptoms, with ultimate recovery. The feeding to chickens of pure cultures of type C in cooked meat medium (as much as would be consumed after about two days without food) has produced typical symptoms of "limberneck" and death, though not in all cases. The effects produced appear to be due to the amount of toxic material consumed. A nonfatal dose produces symptoms of leg weakness, with drooping of wings and tail. A dose which is large enough to be acutely fatal produces severe prostration, all of the muscles being relaxed, the bird decumbent and unable to stand or to raise the head.

Comparative susceptibility of guinea pigs, mice, rabbits and chickens to type A toxin.—A type A toxin, whose minimal lethal dose for guinea pigs was 0.000003 g., was tested to determine the relative susceptibility of the other animals.

The results of the intraperitoneal test on mice was as follows:

Susceptibility of mice to toxin.

Mouse.	Toxin.	Approximate M. L. D.'s for guinea pig.	Symptoms.	Death.
	g.			
2097	0.00032	100	1 hour	5 hours.
2098	.00016	50	1½ hours	5½ hours.
2099	.00008	25	do	6 hours.
2100	.00004	12	2 hours	Do.
2101	.00002	6	do	7 hours.
2102	.00001	3	do	8 hours.
2103	.000005	1.5	4 hours	21 hours.
2104	.0000035	1.2	do	Do.
2105	.000003	1	5 hours	28 hours.
2372	.000002	.6	6 hours	25 hours.
2373	.000001	.3	do	28 hours.
2374	.0000005	.15	do	75 hours.
2375	.0000003	.1	23 hours	Survived but showed symptoms.
2376	.0000002	.06	None	Survived.
2377	.0000001	.03	do	Do.

The results with rabbits, inoculated subcutaneously, were as follows:

Susceptibility of rabbits to toxin.

Rabbit.	Weight.	Toxin.	Approximate M. L. D.'s for guinea pig.	Symptoms.	Death.
	g.	*g.*			
2091	2,000	0.00004	12	42 hours	65 hours.
2092	1,965	.00002	6	65 hours	4 days 17 hours.
2093	1,830	.00001	3	73 hours	7 days 17 hours.
2094	1,750	.000005	1.5	None	Survived.
2095	1,730	.0000035	1.2	do	Do.
2096	1,607	.000003	1	do	Do.

The control test on guinea pigs with the same toxin was as follows:

Guinea pig.	Weight.	Toxin.	Symptoms.	Death.
2626	255	0.00000306	18 hours	5 days, 3 hours.
2627	290	.00000348	do	89 hours.

A test on chickens previously referred to showed that a dose of 0.0006 g. (200 guinea pig M. L. D's) inoculated subcutaneously caused symptoms of "limberneck" and the death of a chicken in 76 hours.

The susceptibility of these 4 animals considered to type A toxin may therefore be shown thus:

	Approximate weight.	Minimal fatal dose
	g.	
Guinea pig	250	0.000003 g.=0.000000012 per gram.
Mouse	20	0.0000005 g.=0.000000025 per gram.
Rabbit	2,000	0.00002 g.=0.00000001 per gram.
Chicken	1,500	0.0006 g.=0.0000004 per gram.

The comparative results show slight difference in the susceptibility of the mouse, guinea pig, and rabbit, when the weight of the animal is considered. The chicken is about 40 times as resistant as the guinea pig to type A toxin. The relatively high susceptibility of the hen to type A botulinus toxin in comparison with tetanus toxin is of interest. In the results published by Knorr a gram of hen requires 100,000 times as large a dose of tetanus toxin to bring about fatal results as a gram of guinea pig. The horse in accordance with the results of Hart and Hayes is probably the most susceptible animal. A dose of about 300 guinea pig minimal lethal doses was a lethal dose for a horse which in a 500 kg. horse would represent a susceptibility for weight approximately 7 times as great as that of the guinea pig.

As to the practical application of the results of these tests it will be noted that while the guinea pig, rabbit and mouse differ little in their susceptibility per gram weight, the symptoms develop much more rapidly in mice inoculated intraperitoneally than in guinea pigs and rabbits inoculated subcutaneously. The symptoms are also more characteristic in mice inoculated intraperitoneally as has been previously pointed out (Bengtson, 1921, *b*).

It is also possible to get results with smaller amounts of the toxin as is shown by the fact that symptoms were obtained in a mouse inoculated with one-tenth the amount fatal to a guinea pig. In the testing of suspected food substances, the mouse therefore offers an advantage over other animals.

Susceptibility of man.—Van Ermengen (1897) calculating on the basis of the dose of toxin fatal to a rabbit estimated that one-thirtieth mg. of dried toxin injected subcutaneously would be a fatal dose for a man of 70 kg. weight. Brieger and Cohn estimated that if 0.00000005 g. of their strongest tetanus toxin was fatal to a mouse weighing 15 g., the fatal dose for a 70 kg. man would be 0.00023 g. or about one-fourth of a mg. The strongest toxin produced by the writer was a type B toxin the fatal dose of which for a 250 gram guinea pig was 0.00000003 g. On this basis the fatal dose for a man of 70 kg. weight would be 0.0000084 g. or approximately one one hundred twentieth mg. It therefore appears that the toxin of *C. parabotulinum* may be considerably more toxic than that of *C. tetani*.

Relationship of size of dose of toxin when injected and when fed.—Lippmann found the minimal lethal dose of a toxin for the white mouse to be 0.000025 c. c. when injected subcutaneously and 0.04 c. c. when fed; the minimal feeding dose being therefore 1,600 times the subcutaneous dose. Bronferbrenner and Schlesinger (1922, *a*) state that the relation between the size of the minimal lethal dose by mouth and by intraperitoneal inoculation of crude filtrate may be represented by the ratio 1,000:1. If the toxin is precipitated with ammonium sulphate and the precipitate redissolved (presumably in salt solution)

the relation between the lethal dose by mouth and by intraperitoneal inoculation becomes 100,000:1. These are all much higher ratios than those found by the writer in the case of chickens (p. 67).

Absorption of the Toxins by the Tissues and the Possibility of Its Elaboration in the Animal Body.

This group of organisms is unique in that toxins are produced which in contrast to all other recognized soluble exo-toxins, are absorbed in the digestive tract. In experimental work on animals it is easily demonstrated that the toxin is injurious also when injected, in fact it is much more toxic by this route than when absorbed in the digestive tract. Though there is not recorded in the literature any evidence that botulism has ever occurred in man as the result of introduction of the toxin by the subcutaneous route, it may be inferred by analogy that a sufficient amount of toxin introduced by this route would be equally and very probably more highly fatal than when taken by mouth. Evidence of the multiplication of the organism with the production of toxin in wounds is lacking, e. g., in the extensive investigation of the flora of war wounds undertaken during the World War, the isolation of the organism is not recorded.

The explanation of the harmful effect of the toxin via the digestive tract in contrast to the inocuousness of tetanus toxin has been sought in tests undertaken to determine the effects of the digestive juices as well as other investigations on the properties of the toxin.

Bronfenbrenner and Schlesinger (1921) found that the toxin was not digested by either pepsin or trypsin. Schübel confirmed this. Pepsin H–Cl did not destroy the toxin after acting for 4 hours on it at a temperature of 38° C. using the pepsin H–Cl in the proportion of 1 part to 4 parts of the toxin. Trypsin in alkaline solution allowed to act for 4 hours also failed to destroy the toxin.

The dialyzability of the toxin may be considered in relation to its ready absorption through the digestive mucosa. Van Ermengem (1897) reports that the toxin dialyzed slowly. It was only after 18 to 20 hours that sufficient toxin passed through a membrane to kill rabbits on doses of 5 c. c. of the dialysate (100 c. c. volume), the minimal lethal dose of the toxin being 0.001 c. c. originally. Schübel, in attempting to dialyze out ammonium sulphate with which he had precipitated the toxin, found that the toxin had completely disappeared from the inner container. The toxin in the fluid form also passed through parchment and collodion bags. Bronfenbrenner and Schlesinger (1922, *b*) found that, although a crude toxic filtrate dialyzed with comparative ease, an acidified filtrate whose toxicity was enormously increased, was retained in the dialyzing bag.

The indications are that absorption through the digestive mucosa is much more rapid than can be shown in dialyzing experiments and is dependent on a different mechanism.

Bronfenbrenner (1922, *a*) suggests that the great resistance of the toxin to acids and its instability in alkalies point to an absorption through the stomach and upper duodenum which are acid rather than through the intestine which is alkaline in reaction. Experiments indicate that active digestion increases the potency of the toxin presumably due to the acid reaction at the time. Van Ermengem (1897) showed that if 5 c. c. of the toxin were introduced into the stomach of a guinea pig, sufficient toxin was present in the circulating blood to kill a mouse on a dose of 0.5 c. c. 30 minutes after the inoculation of the toxin but not 15 minutes after. Forssman found that there was no absorption of the toxin when directly introduced into the caecum or large intestine, and that in test tube experiments 10 grams of the caecal contents of a rabbit would precipitate 1,000 minimal lethal doses of toxin. When the toxin was introduced through a catheter or by laparotomy into the small intestine the same symptoms were produced as by subcutaneous inoculation, but larger doses were required.

Absorption through mucous or serous membranes other than those of the digestive mucosa has not been demonstrated, except in the case of intraperitoneal and intrapleural inoculations when it appears that a local effect is produced on the diaphragm due to the absorption of the toxin, as pointed out by Forssman. Van Ermengem (1897) carried out tests on rabbits and cats to determine whether the toxic filtrate from the ham causing the Ellezelles outbreak could be absorbed by the conjunctival mucous membranes and negative results were obtained. A concentrated filtrate from a pure culture instilled into the eye of a cat also failed to produce any effect.

Possibility of multiplication of spores in the body.—The question as to whether it is possible for toxin-free spores to multiply in the body of man and animals and to produce toxin has been considered by van Ermengem (1897) and other European investigators. Van Ermengem was of the opinion that the organism was not capable of multiplying in the body, basing his conclusions on the fact that the organism failed to grow characteristically or to produce toxin at body temperature and that the same effects were produced in animals whether cultures or filtrates were used. Cultural and microscopic tests were also made which tended to show that the organism did not multiply in the organs (liver, spleen, kidneys, or salivary glands). The organism when inoculated incited an active phagocytosis and disappeared rapidly from the site. The same opinion was later expressed by Landmann and Römer.

In this country the question has received considerable attention, particularly from the standpoint of possible danger of botulism through multiplication of spores which may be present in the food eaten. Armstrong, Story, and Scott obtained positive results with washed spores but found that spores heated to a temperature of 80° C. for 30 minutes failed to kill guinea pigs when injected subcutaneously. Heated cultures which were force fed on grass and feed also failed to produce symptoms in guinea pigs.

Edmondson, Giltner, and Thom, following Bullock and Cramer, who worked with tetanus spores, found that symptoms of botulism could be induced in animals by injecting a sufficient number of spores with a certain amount of calcium chloride. It was also shown that some of the control pigs receiving spores without calcium chloride developed typical symptoms and died. Toxin-free spores of the Boise strain when fed in sufficiently large doses also produced symptoms of botulism and death. In these tests the spores were subjected to a temperature of 80° C. for 15 minutes. Orr (1922) states that the optimum temperature for the growth of 16 strains which he tested was that of body temperature. He showed that experimental botulism could be produced in laboratory animals by inoculating or feeding massive quantities of toxin-free spores and the spores could be recovered from the spleen and liver. Orr heated his spores in a water bath at 80° C. for 30 minutes.

Burke, Elder, and Pischel, reviewing the evidence for and against infection occurring in human beings through the ingestion of toxin-free spores, with the subsequent elaboration of toxin sufficient to produce the disease, present the following arguments against the theory:

> (1) There should be recorded outbreaks of botulism without a history of spoiled food and (2) without a history of preserved food. (3) It should be possible to demonstrate experimentally an infection following the ingestion of toxin-free organisms. (4) There should be more cases of botulism, unless an extremely large number of organisms are necessary for the development of an infection. (5) Outbreaks of botulism should follow the eating of thoroughly cooked spoiled preserved food.

They conclude that the evidence is against the possibility and consider it doubtful whether toxin-free organisms ever occur in heated or unheated foods in sufficiently large numbers to cause the disease.

Hall and Davis were unable to confirm completely the results obtained by Edmondson, Giltner and Thom, and Orr (1922). Calcium chloride administered subcutaneously, intraperitoneally, or intravenously had no effect upon the production of botulism following the intraperitoneal inoculation of guinea pigs with toxin-free spores of *C. parabotulinum*, the dose of spores used being the maximum non-symptomatic dose, although concentrated suspensions of spores detoxified by heat (80° C. for 15 minutes) were able to produce the dis-

ease when given intraperitoneally in suitable doses, but not when given by mouth.

The results of Edmondson, Giltner and Thom, and Orr were confirmed as regards multiplication of detoxified spores with development of symptoms of botulism in animals on injection but not on feeding. The conclusion reached by Hall is therefore "that the possibility of human disease contracted by eating freshly cooked food contaminated with *Bacillus botulinus* would be slight."

Coleman and Meyer fed and inoculated guinea pigs with spores detoxified by heat (80° C. for 1 hour) but they found it necessary to employ considerably larger doses than those used by other investigators in order to cause symptoms or death. They consider that they were able to demonstrate the germination of detoxified spores by injecting spore suspensions subcutaneously into the tied vein of rabbits or into the anterior chamber of the eye of a rabbit, and subsequently recovering vegetative forms. The centrifugalized blood serum of the rabbits which had received an emulsion of detoxified spores in a tied loop of the jugular vein was found to be toxic for mice, indicating a multiplication of the organisms with the development of toxin. In conclusion, the authors state that though they consider that the germination of toxin-free spores in the animal body has been demonstrated beyond doubt, it should not in any way be taken as a criterion for what may take place in the human body by the consumption of spores of the organism in the natural way.

The comment may be made on the foregoing review of the literature of the subject, that though there is some evidence that infection has been experimentally produced in laboratory animals, there is as yet no definite basis for assuming that such infection ever has been brought about under natural conditions in human beings but rather the evidence is against such a possibility. Most of the workers agree that though botulism occurring as the result of the ingestion of toxin-free spores has not been proven, the possibility should be considered. It must be true that a few toxin-free spores ingested with foods are not harmful, since, owing to the widespread occurrence of the organism in the soil, foods (not preserved foods) must necessarily be frequently contaminated with it and cases of botulism do not occur under such circumstances. However, the question as to whether preserved foods in which the toxin has developed and which have been heated, prior to consumption, to a temperature which has destroyed the toxin but which presumably still would allow a large number of viable spores to remain, are harmful or not is not so easily answered. The matter of the great variation in thermal death points of different strains of organisms must be considered in drawing conclusions in cases which have been cited to prove that the

harmful properties of food containing botulinus toxin have been destroyed by heating. As pointed out previously, several of the strains which have been concerned in human outbreaks have had a comparatively low thermal death point (below 95° C. for 10 minutes) and it is probable that heating of contaminated foods at times destroys the spores as well as the toxin. It is of interest in connection with this discussion that the organism of van Ermengem was of the nonproteolytic variety. The proteolytic types found in this country with which all the experimental work along this line has been carried out, have a greater growth propensity and there may be a difference between the proteolytic and nonproteolytic types as to their power of invasiveness in animal tissue.

Mode of Action of The Toxin.

Van Ermengem (1897) demonstrated the rapid absorption of the toxin both when inoculated subcutaneously and when fed. Absorption after inoculation was shown in an experiment in which mice were inoculated with toxin at the tip of the tail. The tail was amputated at the root after 5, 10, 20, 30, and 60 minutes. All of the mice which had the tail amputated after a longer period than 10 minutes after the inoculation died, while the others survived. Schübel determined the rapidity of absorption by injecting a certain amount of toxin into the dorsal lymph sack of the frog and then removing it after varying intervals. Even after 2 minutes a sufficient amount was absorbed to cause symptoms in from 15 to 18 hours.

The rapidity of absorption through the digestive mucosa has already been referred to (p. 73).

That the toxin may be present in the blood in appreciable quantities was shown by Kob, who produced typical symptoms in mice by inoculating with the blood from a patient 9 days after the poisonous food was eaten. It has been shown experimentally by the writer (Bengtson, 1921, *b*) that the toxin is present in the circulating blood of animals fed with toxin. Blood from a guinea pig fed the day previous with toxic cultures, contained sufficient toxin to produce symptoms of botulism and death of mice with 1.0, 0.5, and 0.1 c. c., but not with 0.01 c. c. or smaller amounts of the blood, while 0.5 c. c. of the serum produced late symptoms and death.

The fate of the toxin after it enters the blood stream is not known. Investigations on this phase of the subject are not as numerous as those which have been undertaken in the case of tetanus toxin. It is evident, however, that the toxin has a particular affinity for nerve tissue, just as is true of tetanus toxin.

Kempner and Schepilewsky state that 1 c. c. of brain emulsion would neutralize 3 to 4 minimal lethal doses of toxin if mixed *in*

vitro. They also found that the material from the central nervous system of an acutely poisoned guinea pig did not possess the slightest toxicity. This material possessed the same property of neutralizing a certain amount of toxin as that from normal guinea pigs.

Tests also made by Kempner and Schepilewsky showed that liver, kidney, spleen, and muscle tissue did not adsorb toxin. Schübel showed that these same tissues, as well as the heart, stomach, and lungs, failed to adsorb the toxin when permitted to act for three hours.

Just how the toxin reaches the nerve centers and what are the effects produced there have, however, not been determined definitely. The question as to whether the distribution of the toxin is central in origin or whether (as seems to be true of tetanus toxin) it is diffused centripetally to the central nervous system from the peripheral nerves has not been answered.

That the toxin is transported by other means than the blood stream has been shown by Schübel. This was shown by the following experiment: A method was devised for measuring the injury of the toxin on the ischiadic nerve by faradic currents and the determinations made for a given amount of toxin. If the blood supply through the *arteria iliaca* were shut off and the animal treated as before, the same effect was produced on the ischiadic nerve. He considers two possibilities for the diffusion of the toxin. It may be that the toxin reaches the nerves by way of the lymph channels, and by penetration into the nerve sheath completely poisons it after a time. This hypothesis is supported by the fact that the toxin may be demonstrated in the oedematous fluid. The other possibility is that the toxin (presumably having reached the central nervous system) produces injury to the ganglion cells of the anterior horns of the cord, so that certain chemical changes are produced. The toxin as such, or through decomposition products formed by it, may progress away from the nerve cells through changes in the osmotic pressure or by diffusion peripherally in the neurones and so bring about the complete immobility of the nerve fibers. The action of the muscles would then be rendered impossible in an indirect way. It was also found by Schübel that there was no deterioration of the muscle tissue in frogs, that the muscles were unchanged and not atrophied. The muscle could be stimulated by weak galvanic currents, reacting with a prompt contraction.

The pathologic effects produced on nerve tissue have been considered by a number of workers, including van Ermengem (1897), Marinesco, Kempner and Pollack, Ossipoff, Römer and Stein, Bürger, Paulus, Semerau and Noack, Schübel, and Ophüls. Dickson (1918, *a;* 1923) has reviewed the work of these authors and reference

may be made to his publications for them. He has also made a further study of the subject himself.

The matter of injury to the nerve tissues as evidenced by histological changes has been considered by most of the European authors in explaining the effects produced. There is a rather general agreement that histological changes are produced, but there is lack of uniformity in the results obtained in these investigations. The reason for these differences may be ascribed to variations in the effects produced according to the acuteness of the symptoms, the species of animal and other factors. The use of vital staining methods which have been employed in the later investigations have given more definite information as to the histological changes than were obtainable formerly.

Dickson and Shevky approached the subject from a physiological point of view and studied the changes brought about in the functioning of the nerve tissues rather than changes in the structure. The plan of their investigations was "to obtain a number of quantitative physiological observations concerning the conduction of nerve impulses along the various nerves of the autonomic system in animals which have been previously poisoned with *botulinus* toxin for comparison with similar data obtained from normal animals. In order to obtain the comparative data we employed the method of quantitative faradic stimulation as developed by Martin in which the threshold of nerve stimulation or the intensity of the stimulus necessary to cause physiological response is stated in what he describes as Z units." Using both normal and botulism animals data were obtained on the threshold of stimulation of the vagus nerves for cardiac inhibition and for the augmentor effect upon the small intestine; of the *chorda tympani* nerve for initiating the flow of saliva; of the pelvic nerve (*nervus erigens*) for initiating movements of the bladder wall and erection of the penis; of the oculomotor nerve for contraction of the pupil. In all of these cases it was shown that a higher intensity of stimulus was required to induce the initial reaction in botulism than in normal animals. Negative results were obtained in tests on the vasomotor and the splanchnic nerves. The conclusion is reached that the effects produced are due to a blocking of the nerve impulse in those portions of the autonomic nervous system described as the bulbosacral and prosomatic outflows of connector fibers, but that there is no damage to the nerves of the thoracicolumbar outflow. The exact location of the damage and the mechanism of blocking the nerve impulses were however not ascertained. The results indicate that the lesions produced are not of central distribution but peripheral.

The pathology of botulism as regards the effects produced on other parts of the body aside from the nerve tissues has been covered in

the monograph of Dickson (1918, *a*) to which reference on this subject may be made. In addition to a review of the data published previously he records his own observations on material derived from guinea pigs, rabbits, cats, dogs, and chickens, and several human cases. He calls particular attention to the formation of thrombi which he considers a characteristic lesion produced by the action of the toxin which has not been reported by other observers.

Regarding the elimination of toxin in nonsusceptible as well as s susceptible animals we have the information that it may be eliminated, in the case of chickens, in the feces. It was shown by Buckley and Shippen that though chickens which eliminated toxin in the feces did not show symptoms of botulism, the droppings when fed to horses caused symptoms and death. These results were confirmed by Thom, Edmondson and Giltner. Van Ermengem (1897) was of the opinion that the toxin was completely destroyed by the cellular elements. By placing a ligature at the base of the ear of a rabbit before inoculating the toxin, no effect was produced if the ligature were not removed for 24 hours. He was not able to show the presence of the toxin in the saliva or urine. Schübel has recently reported, however, that the toxin was eliminated in the urine in the case of frogs, which, on account of respiration through the skin, do not succumb as the result of respiratory failure but may be kept alive for long periods. The elimination took place gradually for periods of weeks or months.

Symptomatology.

The clinical manifestations of botulism in man have been adequately described by Rosenau and by Geiger, Dickson, and Meyer, to whose publications reference may be made for detailed information. The symptoms due to the toxin are those brought about as the result of involvement of certain nerve centers and the most characteristic symptoms produced include great muscular weakness, dimness of vision, diplopia, palpebral ptosis, difficulty in swallowing and talking, constipation, inhibition of secretions, and respiratory paralysis.

The symptoms in laboratory animals are fully described by van Ermengem (1897, 1912). The effects produced in the cat by subcutaneous inoculation were considered by van Ermengem to be more characteristic than those of other animals. He states that the neuroparalytic symptoms produced may be designated as pathognomic in this animal. Mydriasis was very marked, as well as ptosis of eyelids, with staring appearance of the eyes, aphonia, aphagia, prolapse of the tongue, secretion of thick mucus from the mouth, a croupous cough, retention of urine and feces, and general hypotonicity of the voluntary muscles.

Forssman studied the effects produced by inoculation through various routes. The same symptoms were produced whether the

toxin was administered subcutaneously, intracerebrally, subdurally, or through the digestive tract, though much larger doses were necessary by the latter route. A quite different symptom complex was brought about by inoculation into the peritoneum, the pleura, or the lungs. The most prominent symptom in this case was dyspnea. This manifestation appears very late in subcutaneous inoculations. The respiration was distinctly costal and in the guinea pig fell from 120 to 160, to 20 to 30 per minute. Forssman considers that the effect produced is due to a rapidly progressing paralysis of the diaphragm. The symptoms were produced by smaller amounts and also appeared much earlier than when inoculations are made by the subcutaneous route. When large doses (500 to 1,000 minimal lethal doses) were injected, symptoms appeared within 4 hours in the guinea pig.

The writer has found this method of inoculation in mice a very useful one in determining the presence or absence of toxin. Characteristic symptoms of costal breathing occur very early.

Bronfenbrenner and Schlesinger (1922, *a*) report the first appearance of symptoms in 30 minutes and death within 1½ to 2 hours. The time of appearance of symptoms and death of mice, as related to size of dose as observed by the writer, are shown on page 70.

Regarding the symptoms of the guinea pig, which is the animal used at the Hygienic Laboratory in the routine potency testing of botulism antitoxins, the following main points are noted after subcutaneous injection: hypotonicity of muscles, pendulent abdomen, hypersecretion of saliva, and prostration. Hypotonicity of the muscles is the first symptom manifested and is easily determined by lifting the guinea pig. The flaccidity of the muscles is very apparent in comparison with a normal pig. The pendulent abdomen is evidence of relaxation of the abdominal muscles. The hypersecretion of saliva is a very characteristic symptom. If this occurs within the first 48 hours, the pig invariably dies, and usually if it occurs later. Rarely, an animal exhibiting definite symptoms recovers. Prostration is an indication of severe symptoms, and the animal never recovers.

Frequently guinea pigs exhibit less acute symptoms consisting of hypotonicity of muscles, loss of weight, emaciation, and cachexia. These symptoms appear in the course of 4 to 5 days or later. Pigs in this condition may die in the course of 3 or 4 weeks but often recover.

The symptoms are considered sufficiently characteristic for diagnosis as in tetanus and necropsies are not performed in routine work unless there is some question as to the cause of death.

ANTITOXIN.

METHODS OF IMMUNIZATION.

Antitoxins against the three types of organisms may be produced by inoculating animals with increasing doses of toxin. Goats and horses have been found most useful for this purpose. Kempner found immunization of rabbits and guinea pigs difficult. Later, however, he obtained an antitoxin by inoculating goats. Toxin from the van Ermengem culture was inoculated over a period of 6½ months, at the end of which time 1/100,000 c. c. of the serum protected against 1 minimal lethal dose in guinea pigs. He also produced an antitoxin with the strain which he himself isolated from swine feces. The latter serum protected against the van Ermengem strain as well as against his own, indicating the identity of the two strains as regards the type of toxin produced.

Forssman also obtained a potent antitoxin by inoculating goats. His antitoxin was produced by inoculating with a much smaller amount of toxin than had been used by Kempner. The advantage of Forssman's method over Kempner's was made the subject of a study by Forssman and Lundstrom. The intervals between inoculations were longer in Forssman's method (8 to 14 days). The antitoxin curve was determined and it was shown that the amount of antitoxin fell after a subcutaneous inoculation and rose quickly after the fourth to the eighth day, reaching the highest point in 15 days and rapidly falling again. The antitoxin curve for *C. botulinum* therefore lay between the curve for diphtheria antitoxin (with the climax on the ninth or tenth day) (Salomonsen and Madsen) and that of tetanus (with the climax on the seventeenth day after the preceding injection of toxin) (Brieger and Ehrlich).

Leuchs (1910) first used horses for producing antitoxin. One horse received toxin of the van Ermengem strain and the other the toxin of the Darmstadt strain. Tests carried out with these antitoxins demonstrated that each antitoxin was specific for the toxin used in immunizing but failed to neutralize the toxin of the other strain.

Immunization by mouth has been shown by Lippmann. By administering increasing doses of toxin to mice, a protection against 4 minimal lethal doses by mouth was obtained. The immunity produced was however local, as when 1 minimal lethal subcutaneous dose was injected the mice died.

THE TOXIN-ANTITOXIN REACTION.

The nature of the toxin-antitoxin reaction was studied by Madsen (1905, 1909) and Leuchs (1910) and others. Madsen showed that as with diphtheria toxin the range between the L+ and the L° dose was considerable. In testing against 250 minimal lethal doses of toxin the range between no symptoms and slight symptoms was

from 0.002 c. c. of antitoxin to 0.0014 c. c., though there were irregularities between these doses, some animals dying and some showing slight symptoms. He concluded that there was no neutralization *point* in the true sense of the word. Similar results have been obtained by the writer. The following protocol shows the results obtained in attempting to determine the L° dose of a certain type C serum:

G. P.	Weight.	Antitoxin per 250 g.	Toxin per 250 g.	Symptoms.	Death.
1326	270	0.0091	0.0002	90 hours	12 days 7 hours.
1327	285	0.0091	0.0002	4 days 20 hours	10 days.
1328	270	0.0087	0.0002	66 hours	14 days.
1329	285	0.0087	0.0002	89 hours	Survived.
1330	270	0.0083	0.0002	66 hours	5 days 18 hours.
1331	290	0.0083	0.0002	4 days 20 hours	7 days 20 hours.
1332	270	0.008	0.0002	18 hours	Survived.
1333	290	0.008	0.0002	9 days 20 hours	Do.
1334	255	0.0077	0.0002	18 hours	5 days.
1335	280	0.0077	0.0002	do	7 days 6 hours.
1336	250	0.0074	0.0002	66 hours	Survived.
1337	280	0.0074	0.0002	do	5 days 23 hours.
1338	245	0.0071	0.0002	18 hours	4 days 5 hours.
1339	280	0.0071	0.0002	do	10 days 5 hours.
1340	245	0.0069	0.0002	do	4 days 5 hours.
1341	280	0.0069	0.0002	do	89 hours.

Dissociation of toxin and antitoxin.—The reaction between the toxin and antitoxin as in tetanus and diphtheria is a binding action and not a destructive one. The union between toxin and antitoxin becomes closer with time. In the earlier stages, however, dissociation may be brought about by various means, as dilution, addition of acid, introduction into the stomach, etc.

Madsen (1909) showed that in mixtures of toxin and antitoxin it often happens that a diluted fraction of the mixture is more toxic than the mixture itself. Thus, in a mixture of 1 c. c. containing about 2,500 minimal lethal doses of toxin and 0.0013 c. c. of antitoxin dilutions were made from 1/5 to 1/3000. No symptoms were obtained with the undiluted mixture, slight symptoms with 1/5 to 1/10, death from 1/20 to 1/800 and slight symptoms from 1/1,000 to 1/3,000. Similar results have been reported by von Behring in the case of tetanus toxin. The explanation is offered that the neutralized toxin-antitoxin mixture undergoes dissociation on diluting.

Leuchs (1910) also showed that by the addition of acid the separation of toxin and antitoxin could be effected. A mixture of toxin and antitoxin to which a drop of N/1 HCl had been added did not kill animals when inoculated intravenously. After standing for a period of 24 hours the dissociation between toxin and antitoxin had taken place to the extent that the mixture now killed animals on intravenous inoculation.

Dissociation of toxin-antitoxin mixtures in the stomach has also been shown by Leuchs (1912). It was possible to neutralize about one minimal lethal dose by mouth when a relatively large amount of

serum was given. If, however, a dose of 10 minimal lethal doses of toxin were used it was not possible to save the animal no matter how large was the dose of antitoxin.

The following protocol of a test with type A toxin and antitoxin carried out by the writer, illustrates this point:

Dissociation of toxin-antitoxin mixtures.

G. P.	Culture.	Death.
1559	0.5 c. c.	+9 hours.
1560	0.1 c. c.	+20 hours.
1561	0.01 c. c.	+44 hours.
1562	0.5 c. c. mixed with 0.5 c. c. antitoxin and fed.	+20 hours.
1563	0.1 c. c. mixed with 0.5 c. c. antitoxin and fed.	+44 hours.
1564	0.01 c c. mixed with 0.5 c. c. antitoxin and fed.	+20 hours.
1565	0.5 c. c. (0.5 c. c., antitoxin inoculated subcutaneously).	Survived.
1566	0.1 c. c. (0.5 c. c., antitoxin inoculated subcutaneously).	"
1567	0.01 c c. (0.5 c. c., antitoxin inoculated subcutaneously).	"

It is probable that the acidity of the stomach may dissociate the toxin and antitoxin and that the toxin is absorbed more rapidly than the antitoxin. The results indicate that favorable results cannot be expected in the treatment of botulism by serum administered by mouth.

VALUE OF ANTITOXIN PROPHYLACTICALLY AND THERAPEUTICALLY.

The prophylactic value of the antitoxin has been demonstrated by several investigators including Kempner. Kempner first investigated the curative properties of the antitoxin. It was found that the serum administered (subcutaneously) to guinea pigs 24 hours after the toxin and after definite symptoms had developed offered protection to the extent that the pigs thus treated survived exceptionally or died several weeks or months later with symptoms of cachexia, while the control animals died in 48 hours. These results were confirmed by Forssman and Leuchs (1912). Forssman showed that the neutralization of toxin by antitoxin varied according to the method of inoculation. No serum therapy was shown for the diaphragmal type after symptoms were evident.

Dickson and Howitt have recently carried out tests to determine the curative value of American antitoxins on guinea pigs and rabbits. As the results of these experiments it was shown that if slightly more than one test dose of toxin was inoculated (a dose which was fatal in 48 hours) the animals could be saved if the antitoxin were given 18 hours after the toxin, although symptoms had developed. If the dose which killed in 48 hours were inoculated the antitoxin could be inoculated 24 hours later and protection afforded. In another test rabbits were fed with an amount of toxin which was fatal in 45 hours. Antitoxin was administered subcutaneously 6, 12, 18, and 24 hours later, and all the animals survived.

The curative effect of antitoxin in human cases has not been determined definitely. It seems reasonably certain that the antitoxin is effective prophylactically as is tetanus antitoxin. As to effects produced when administered after symptoms develop, no certain statements can be made, owing to the fact that spontaneous recoveries of patients showing definite symptoms of botulism have been recorded. The curative properties are dependent on the length of time elapsing between the consumption of the food and the administration of the antitoxin, and also on the amount of toxin which has been ingested. It has been shown in animals that the rapidity with which symptoms appear is in direct relation to the amount of toxin administered. A guinea pig inoculated with 100 minimal lethal doses will develop symptoms and die within 17 to 18 hours, while one which has received 1 to 2 minimal lethal doses will succumb in four days. Reports have been published which indicate that a certain therapeutic value may be ascribed to the antitoxin. Perhaps the most convincing of these is that of Beall.

The most promising field for the use of the antitoxin is in outbreaks in which certain persons who have consumed food containing the toxin have not yet developed symptoms, or only slight symptoms, while the diagnosis of botulism is certain in others who have eaten of the same food or in chickens which have developed symptoms of "limberneck" from eating the food. In any case, the use of antitoxin is indicated, since it is the only known specific remedy and the possibility always exists that the results may be favorable.

Dickson and Howitt advocated the intravenous administration of large doses of antitoxin according to the following plan: "The usual precautions for the administration of horse serum will be observed and the patient will be tested by intracutaneous injection for evidence of hypersensitiveness. When no hypersensitiveness is shown, the serum will be given at once and will be injected into the veins very slowly, not more than 1 c. c. a minute, until the full amount is given. When hypersensitiveness is shown, preliminary subcutaneous, intramuscular and intravenous injections of 1 c. c. at one-hour intervals will be given, and one hour after the last injection the full amount will be injected intravenously at the rate of not more than 1 c. c. a minute.

The following instructions for the use of the antitoxin have been issued by the Ministry of Health of Great Britain:

"BOTULINUS ANTITOXIC SERUM.

"INSTRUCTIONS AS TO USE.

"1. *Immediate administration.*—The only remedy at present known for botulism is botulinus antitoxin given by injection and even this is unlikely to save life if the disease has progressed to a late stage.

It is therefore of the greatest importance *to give the antitoxin at the earliest possible moment*—i. e., as soon as the earliest symptoms of blurred or double vision, giddiness, ptosis, difficulty in speech, or swallowing suggest the diagnosis of botulism.

"2. *Method.*—The effectiveness of treatment by antitoxin for botulism is so greatly increased by intravenous injection that this method of administration should always be employed (otherwise the intramuscular method should be used). The risk of serious symptoms arising in persons highly sensitive to horse serum must be recognized, and when, in the judgment of the medical practitioner, such is likely to be great, he should administer a preliminary injection of a small quantity of the antitoxin (0.5 c. c. = 8 minims) *subcutaneously*. If this produces clear evidence that the patient is sensitive (that is, if a reaction appears in half an hour) then the antitoxin must be given with particular caution, the first intravenous injection consisting of 0.5 c. c. of antitoxin being diluted with 50 c. c. of saline, and subsequent injections being diluted less and less till the full dose has been administered.

"3. *Dose.*—Only large doses will save acute cases. The intravenous dose should be from 20 to 50 c. c. of the antitoxin, previously warmed to body temperature, and injected very slowly; this amount may be repeated daily if necessary.

"NOTE.—All other persons who have consumed the suspected food but have not yet presented symptoms should be given a prophylactic dose of antitoxin (i. e., 10 c. c.) intramuscularly, to be followed by larger doses intravenously should symptoms appear."

IV. STANDARDIZATION OF BOTULISM ANTITOXINS.[1]

VALUE OF STANDARDS.

The value of standard methods for testing and for stating the potency of diphtheria and tetanus antitoxins is universally acknowledged. Before the work at the Hygienic Laboratory was done,* establishing United States standards for these products, nothing definite was known as to the comparative strength of different lots of antitoxin in this country. Firms manufacturing these serums used different units in expressing the potency of their respective products and different methods of testing so that it was difficult or impossible to compare the strength of one antitoxin with another.

Official standards have been promulgated by the United States Public Health Service and a unit established for measuring the potency of tetanus and diphtheria antitoxin, and the testing of these products has been placed on a very satisfactory basis. A uniform

[1] Revision of paper published in the Amer. Jour. Pub. Health, 1921, 11,352.

* Hygienic Laboratory Bulletin No. 21 and Miscellaneous Publication No. 10, U. S. Public Health Service, Washington, D. C.

method of stating the potency or the number of units contained in a serum per cubic centimeter has been adopted. Serums are required to contain a certain number of units, and immunizing and therapeutic doses are prescribed in terms of the United States units instead of volume. The advantages are so obvious as not to require elaboration.

The method used by the Hygienic Laboratory for the testing of botulism antitoxins types A and B does not differ in principle from that used for measuring the potency of diphtheria and tetanus antitoxins. An arbitrary amount of standard antitoxic serum for each type is used as the unit of measurement for determining the potency of antitoxins of unknown potency. Both the standard antitoxin of known value of which a definite amount is used and the antitoxin under test of unknown value of which varying amounts are used are tested against the same dose of toxin, and by a comparison of the results obtained the potency of the unknown antitoxin may be determined in terms of the standard antitoxin.

TOXIN.

Cultures of *C. parabotulinum* and *C. botulinum* types A, B and C yield strong toxins when grown in dextrose beef infusion broth containing a small amount of veal incubated at 37° C. for a period of 10 to 14 days. An initial reaction of pH 9.5 for types A and B (proteolytic) and pH 8.5 for C and nonproteolytic strains has been found favorable for the production of strong toxin. After autoclaving the reactions should be about pH 8.0–8.5 for the proteolytic and pH 7.0–7.5 for the nonproteolytic. Toxins varying in strength from 0.000,01 to 0.000, 1 c. c. of filtrate as a minimal lethal dose for 250-gram guinea pigs were obtained by the use of this medium. The filtrate was found to be fairly stable and the toxin may be used in this form for testing the potency of unknown antitoxins with proper controls, after an initial period of deterioration. It will be necessary to test any particular filtrate each time it is used against the standard antitoxin unit to determine the "test dose."

In the work of standardization dried toxins were used. Tetanus toxin, though exceedingly unstable and unreliable in the form of filtrate, has been found to be very stable in the dried state, continuing at the same point of toxicity over a period of many years when preserved under proper conditions of cold and protected from light and air. It was apparent that a considerable period of time would be necessary to accomplish the work of standardization and it was desirable that the toxin be stable over at least this period. Therefore, it seemed advantageous to use dried toxins which might be expected to be as stable as dried tetanus toxin has been shown to be. This has been found to be the case and exposure of the dried toxin to adverse conditions of temperature, light, etc., has shown it to be at least as stable as the tetanus toxin and probably more so.

Precipitation of the toxin was effected by the same methods as those used for the precipitation of tetanus toxin. By saturating with ammonium sulphate the toxin was salted out and could be skimmed from the surface. After drying over sulphuric acid it was ground to a fine powder and stored *in vacuo* in the cold and dark.

ANTITOXIN.

For the standard antitoxins the most potent serums of each of the types which were available at the time were mixed with glycerin in the proportion of one part of serum to two parts of glycerin. This was distributed in small glass-stoppered bottles of 7 to 8 c. c. capacity. Antitoxins preserved in this way have been found to be very stable.

Owing to the fact that the potency of the serums used for the standards was variable, it has been found desirable to make the glycerin dilutions of the standard in such a way that the method of making the final dilutions to be used in the tests would be similar in the case of all three of the standards.[6] Each serum is therefore diluted at the Hygienic Laboratory with glycerin, so that 1 unit is contained in 0.4 c. c. Since one-tenth unit is used in the test, 1 c. c. of the standard is diluted at the time of making the test to 25 c. c. with 0.85 per cent salt solution and 1 c. c. of this mixture contains one-tenth of a unit.

STANDARDIZATION.

A dried toxin of each of the types A and B was used as the basis for determining the standard unit. The approximate minimal lethal dose for a 250-gram guinea pig was determined for each toxin and an arbitrary amount of toxin containing about 100 minimal lethal doses was used as a test dose preliminary to establishing the unit. The smallest amount of glycerinated serum of each type, which when mixed with this test dose of its corresponding toxin and injected into a 250-gram guinea pig, caused the death of the guinea pig within 96 hours, was considered to contain one-tenth of a unit; a unit of the standard antitoxin, therefore, just fails to neutralize approximately 1,000 minimal lethal doses of the original dried toxin. In all tests if guinea pigs weighing more than 250 grams are used, the doses of toxin and antitoxin are increased in proportion to the weight.

TESTING.

In carrying out a test to determine the potency of an antitoxin of unknown value the standard antitoxin is used as the basis of measurement. The test dose of toxin used in establishing the standard in the Hygienic Laboratory as stated above, was approximately 100

[6] The unit of the type B antitoxin, which was formerly 0.008 c. c., has been diluted further with glycerin in accordance with the above, so that 1 unit is now contained in 0.4 c. c., and it is only necessary for the laboratory using this to dilute to one twenty-fifth with salt solution.

minimal lethal doses of the dried toxins originally used, but in carrying out tests in which other toxins are used it will be necessary to determine the test dose by titrating the particular toxin against one-tenth the standard unit of antitoxin. The dose will probably vary from 50 to 200 minimal lethal doses, since the combining power of different toxins is variable. The test dose of toxin will be that amount which when mixed with one-tenth the standard antitoxin unit and injected into a 250 gram guinea pig just fails to be neutralized by this amount of antitoxin, as shown by death within 96 hours. The test dose of toxin having been established, the number of units in the antitoxin of unknown value is determined by testing varying amounts of antitoxin against the "test dose" of toxin. The smallest amount of antitoxin for human use which when mixed with the test dose of toxin is sufficient to *save* the life of a 250-gram guinea pig is considered to contain one-tenth of a unit. From this the number of units per cubic centimeter may be calculated. Control animals are always inoculated at the same time with the same dose of toxin mixed with one-tenth of the standard unit and this amount of the standard should just fail to neutralize the dose of toxin.

Since the different types of *C. botulinum* and *C. parabotulinum* produce toxins which appear to be entirely specific, it is necessary to test a serum of one of the types against the homologous type only. A "polyvalent" antitoxin is tested for its neutralizing power against the toxin of the types used in the production of the antitoxin.

The potency of the antitoxins submitted for test has varied considerably. A potency as high as 2,500 units of type A per cubic centimeter was shown in one product. This was a "polyvalent" serum and contained also about 75 units of type B antitoxins. Serums testing as high as 200 units per cubic centimeter are considered satisfactory antitoxins.

The method for carrying out the test is similar to that used for the testing of tetanus antitoxins with slight differences. Salt solution of a neutral reaction is used for making dilutions. The toxin and antitoxin are both diluted in such a way that the volume of the amounts used shall be between 1 and 2 cubic centimeters in order that the combined volumes may not be more than 4 cubic centimeters. In the case of the toxin amounts in the neighborhood of 0.02 g. are weighed out and the dilution made according to the weight of the toxin. The dilutions of antitoxin are made by measuring 1 cubic centimeter with a capacity pipette, making this up to the required dilution with the salt solution, and washing out the pipette thoroughly with the salt solution used in making up the dilution. Further dilutions are made from this first dilution if necessary. Measurements of the toxin and antitoxin dilutions are made with delivery pipettes into the inoculating syringes and the mixtures allowed to stand for one hour at room termperature.

Inoculations are made subcutaneously into guinea pigs, the mixture in the syringe being rinsed out with sufficient salt solution to make the total volume injected 4 cubic centimeters.

The following is a sample of a test carried out on a type B antitoxin:

Test of potency of type B antitoxin.

Guinea pig.	Weight.	Toxin.				Antitoxin.					
		Toxin B (test dose per 250 g.)	Actual dose.	Dilution.	Amount of dilution.	Antitoxin per 250 g.	Actual dose.	Dilution.	Amount of dilution.	Symptoms (hours).	Death (hours)
	g.	*g.*	*g.*		*c. c.*	*c. c.*	*g.*		*c. c.*		
2578	245	0.000063	0.000062	1/20,000	1.23	0.002	0.00196	1/1,000	1.96	None.	Survived.
2579	245	.000063	.000062	1/20,000	1.23	.001	.00098	1/1,000	.98	24	74
2580	250	.000063	.000063	1/20,000	1.26	.0005	.0005	1/1,000	.5	17	23
2581	265	.000063	.000069	1/20,000	1.34	.00025	.00027	1/1,000	.27	17	23
Controls.											
2586	250	.000063	.000063	1/20,000	1.26	.04	.04	1/25	1.0	24	74
2587	250	.000063	.000063	1/20,000	1.26	.04	.04	1/25	1.0	24	64
2588	260	.000063	.0000655	1/20,000	1.31	.04	.0416	1/25	1.04	24	74
2589	270	.000063	.000068	1/20,000	1.36	.04	.0432	1/25	1.08	24	94

The results of this test show that the amount of antitoxin which will protect against the test dose of toxin lies between 0.002 and 0.001 c. c. Another test was therefore put on with doses of antitoxin at closer intervals.

Guinea pig.	Weight.	Toxin.				Antitoxin.					
		Toxin B (test dose per 250 g.).	Actual dose.	Dilution.	Amount of dilution.	Antitoxin per 250 g.	Actual dose.	Dilution.	Amount of dilution.	Symptoms (hours).	Death (hours).
	g.	*g.*	*g.*		*c. c.*	*c. c.*			*c. c.*		
2602	270	0.000063	0.000068	1/20,000	1.36	0.002	.00216	1/1,000	2.16	None.	Survived.
2603	245	.000063	.000062	1/20,000	1.23	.002	.00196	1/1,000	1.96	None.	Survived.
2604	255	.000063	.000064	1/20,000	1.29	.0013	.00133	1/1,000	1.33	65	113
2605	245	.000063	.000062	1/20,000	1.23	.0013	.00127	1/1,000	1.27	65	71
2606	255	.000063	.000064	1/20,000	1.29	.001	.00102	1/1,000	1.02	17	53
2607	245	.000063	.000062	1/20,000	1.23	.001	.00098	1/1,000	0.98	17	50
Controls.											
2612	245	.000063	.000062	1/20,000	1.23	.04	.0392	1/25	0.98	24	64
2613	265	.000063	.0000668	1/20,000	1.34	.04	.0424	1/25	1.06	24	50
2614	285	.000063	.0000718	1/20,000	1.44	.04	.0456	1/25	1.14	24	74
2615	295	.000063	.0000743	1/20,000	1.49	.04	.0472	1/25	1.18	24	74

Since 0.002 c. c. protected against the test dose of toxin, this amount of antitoxin represents approximately one-tenth unit. One unit would therefore be contained in 0.02 or 1 c. c. would contain 50 units. The potency of this antitoxin is about 50 units per c. c.

DEFINITION OF UNIT.

A condensed statement of the definition of the unit and method of testing is appended:

The unit of botulism antitoxin (type A) is a definite amount of the standard antitoxin; for the standard antitoxin in the concentration used at present, and to be sent out to serum establishments which may be licensed for the production of botulism antitoxin, the unit is 0.4 c. c. of the glycerinated solution. The antitoxin to be tested is compared with the standard unit by means of a test dose of toxin; this test dose of toxin is chosen so that when mixed with one-tenth of a unit of the standard antitoxin and injected subcutaneously into a 250-gram guinea pig, the guinea pig will die in about 96 hours. That amount of the unknown antitoxin which when similarly mixed with the test dose of toxin and injected is just sufficient to protect a 250-gram guinea pig contains one-tenth of a unit. The test dose of toxin will usually be found to contain from 50 to 200 minimal lethal doses. Before injection the doses of toxin and antitoxin are to be thoroughly mixed, and then kept at room temperature for one hour to allow combination to take place. The guinea pigs should be between 250 grams and 300 grams in weight, and the doses of toxin and antitoxin are to be increased proportionately to the weight for guinea pigs weighing over 250 grams. With every test of an unknown antitoxin, four control guinea pigs should receive the test dose of toxin mixed with the test dose (one-tenth unit) of standard antitoxin, so that the two antitoxins may be accurately compared and the test dose of toxin increased if necessary in subsequent tests; liquid toxins have been found to decrease gradually in toxicity. In diluting the standard antitoxins, the method indicated on the label should be followed. The first dilution should be made with a capacity pipette, to be washed out in the diluting fluid, on account of the viscosity of the glycerinated serum.

The units of botulism antitoxins (types B and C) are similarly definite amounts of the standard types B and C antitoxins to be sent out to serum establishments which may be licensed for the production of these antitoxins, the unit of each being 0.4 c. c. of the glycerinated solution. Antitoxins to be tested are compared with the standard units by means of test doses of types B and C toxins, using the same method as above described for type A.

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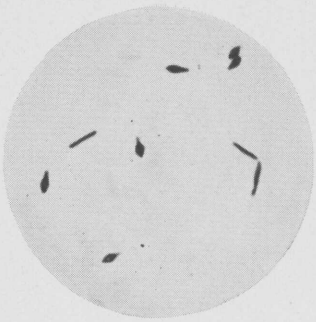
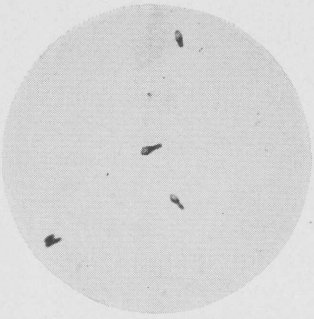


Fig. 1



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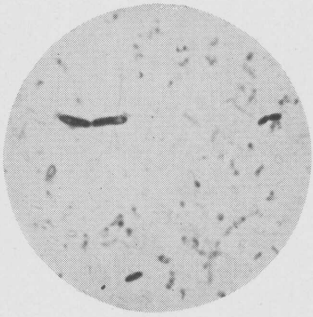

Fig. 3

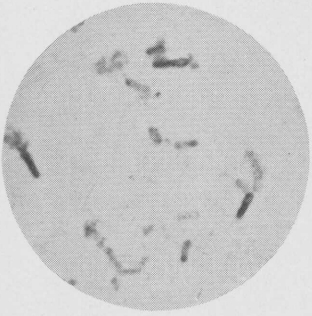

Fig. 4

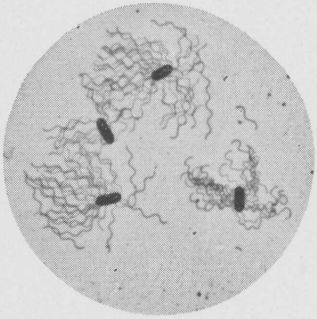

Fig. 5

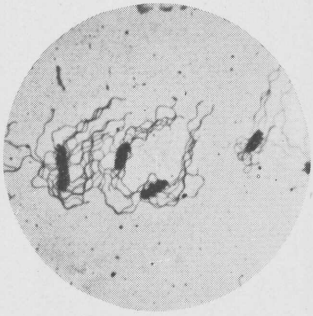

Fig. 6

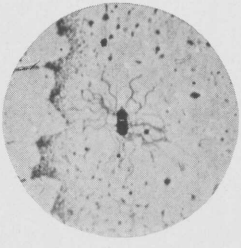

Fig. 7

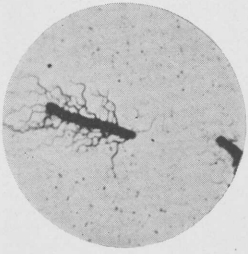

Fig. 8

PLATE I.

FIG. 1.—Spores, *Clostridium parabotulinum* type A, Strain 27 (Canton). 48-hour culture in cooked meat medium, Gram stain.

FIG. 2.—Spores, *Clostridium parabotulinum* type B, Strain 5 (Nevin). 48-hour culture in cooked meat medium, Gram stain.

FIG. 3.—Spores, *Clostridium botulinum* type B, Strain 21 (Lister Institute Strain 21). 48-hour culture in one-tenth per cent agar.

FIG. 4.—Spores, *Clostridium botulinum* type C, Strain 7 (Saunders). 48-hour culture in cooked meat medium.

FIG. 5.—Flagella, *Clostridium parabotulinum* type A, Strain 1 (Memphis). Casares Gils stain.

FIG. 6.—Flagella, *Clostridium parabotulinum* type B, Strain 5 (Nevin). Casares Gils stain.

FIG. 7.—Flagella, *Clostridium botulinum* type B, Strain 21 (Lister Institute Strain 21). Casares Gils stain.

FIG. 8.—Flagella, *Clostridium botulinum* type C, Strain 7 (Saunders). Casares Gils stain.

HYGIENIC LABORATORY BULLETINS OF THE PUBLIC HEALTH SERVICE.

The Hygienic Laboratory was established in New York, at the Marine Hospital on Staten Island, August, 1887. It was transferred to Washington, with quarters in the Butler Building, June 11, 1891, and a new laboratory building located in Washington, was authorized by act of Congress March 3, 1901.

Of the bulletins published by the laboratory since its establishment copies of the following are available for distribution and may be obtained without cost by applying to the Surgeon General, United States Public Health Service, Washington, D. C.

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No. 43.—The standardization of tetanus antitoxin (an American unit established under authority of the act of July 1, 1902). By M. J. Rosenau and John F. Anderson.

No. 65.—Facts and problems of rabies. By A. M. Stimson.

No. 73.—The effect of a number of derivatives of choline and analogous compounds on the blood pressure. By Reid Hunt and R. de M. Taveau.

No. 78.—Report No. 4 on the origin and prevalence of typhoid fever in the District of Columbia (1909). By L. L. Lumsden and John F. Anderson. (Including articles contributed by Thomas B. McClintic and Wade H. Frost.)

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