# THE PATHOGENICITY OF THE SPORES OF *CLOSTRIDIUM BOTULINUM*

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(With Plate 5)

## INTRODUCTION

Several attempts have been made over a period of years to define the direct capabilities of *Clostridium botulinum* as an infective agent, in distinction to its indirect action in rendering foodstuffs highly dangerous by virtue of the toxin it can produce therein under certain conditions.

Van Ermengem (1897), who first described the species, concluded that in clinical cases the organism played no part in the disease and that when it was isolated from the tissues it had been present as a saphrophyte. Other workers later used guinea-pigs and occasionally mice in experiments in which botulinum spores after being heated to a varying degree were administered by injection or feeding. Thom, Edmondson & Giltner (1919) and Edmondson, Giltner & Thom (1920) produced fatal botulism in guinea-pigs with subcutaneous injections of 30 million viable spores, and sometimes with oral doses. The local use of calcium salts was of value in producing the end result. Geiger, Dickson & Meyer (1922) emphasized the need for careful detoxification of spore suspensions to exclude effects from extraneous toxin. Orr (1922) showed that body temperature was optimum for toxin production, also that the subcutaneous injection of some strains killed guinea-pigs and mice in doses of 50 and 30 million viable spores respectively. Because the spores had been heated at 80° C. for 30 min. it was postulated that the fatal botulism arose from toxin elaborated in the animals on the germination of the spore inoculum. Coleman & Meyer (1922) claimed that only 'enormous' numbers of spores such as 180 million viable spores subcutaneously or several billion orally could kill guinea-pigs. They proved that germination of spores did occur in experimental animals and concluded that the botulism eventually induced in experimental animals was much more likely to result from the active growth of the injected spores, than from the release of any pre-formed toxin present in the latter. Calcium given separately from the spore injection failed to induce germination in the experiments of Hall & Davis (1923), who doubted whether spores could be induced to germinate *in vivo* and held that botulism in experimental animals resulted from extraneous toxin in the inoculum. Starin & Dack (1925), too, injected calcium separately from the spores and failed to induce germination. By giving subcutaneously very large numbers of spores such as 60 million to guinea-pigs and 15–25 million to mice these authors could produce fatal botulism. Coleman (1929) successfully produced the disease in guinea-pigs with small numbers of spores

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which were shown to germinate when injected into muscle which had a few hours previously been devitalized with 0·6–0·8 ml. of 10 % formalin solution.

In the majority of cases, therefore, earlier workers have produced the botulism syndrome in experimental animals from injections of large numbers of apparently toxin-free spores. The evidence is conflicting as regards the source of the toxin which produces the fatal result.

This paper describes some experiments in mice in which a quantitative study has been made of the effect of intramuscular injections of botulinum (type A) spores both alone and together with a calcium salt.

## METHODS

### *Strains*

Two type A strains, namely 4587 and Hall were used. Both are highly toxic strains, being capable of producing $1 \times 10^6$ mouse LD 50/ml. under suitable conditions. Stock cultures were kept at 0° C. in sealed tubes of cooked meat medium.

### *Production of spores*

A search was instigated to find a medium on which *Cl. botulinum* would produce a high proportion of spores, as the yield from cooked meat medium or broth was inadequate. Solid media such as dorset egg, Loewenstein, solid serum, and Loeffler solid serum were tested, but only from the last named was the relative proportion of spores to bacilli sufficiently great. The Loeffler cultures also gave the cleanest product for harvesting as it consisted of a clear yellow fluid containing a deposit having upwards of 85 % spores. Batches of the medium in tubes or bottles were inoculated with a few drops of young cooked meat culture and incubated in anaerobe jars at 37° C. for 3–6 weeks. The contents of the tubes were pooled and filtered through glass-wool to remove coarse debris, and the spores were washed with distilled water by repeated centrifugation. The final deposit after at least three washings was resuspended in distilled water to give a suspension of suitable density. It was found that the liquor from the liquefied Loeffler cultures had 100,000 mouse LD 50 per ml., but the toxicity of the supernatant from the first and second washings was only 1000 LD per ml. The supernatant discarded after the third washing was only toxic when 0·1 ml. was injected, and after the first of the heat-treatments of 80° C. for an hour, the distilled water suspending the stock suspension was non-toxic in a volume of 0·5 ml. In no instance during the periodic testing of the suspending fluids of the several stock spore suspensions was toxin ever shown to have developed during their storage in the refrigerator.

### *Total count*

The direct or total count of spores suspended in distilled water was made in a Levy counting chamber. The spore suspensions were suitably diluted to give about 100 spores distributed over each set of 80 small squares. For each count at least 240 and often all of the 400 small squares were examined.

*Viable count*

The method of Miles & Misra (1938) for surface growth on replicated plates was used. The medium finally selected as giving the highest viable counts was tryptic meat-broth agar enriched with 0·5 % glucose and 3·0 % Fildes peptic digest of blood. The plates were dried in an oven at 60° C. for 1½ hr. A known volume of a suitable dilution of spores was delivered in six drops from a standardized dropping pipette and allowed to spread over the surface of the agar. The plates were incubated for 18 hr. at 37° C. in an atmosphere of 5 % carbon-dioxide-hydrogen gas in anaerobe jars, by which time all the colonies were of a size easily visible to the naked eye.

*Mice*

White mice largely from one source and weighing 18–21 g. were used. Intramuscular injections in a volume of 0·1 ml. were given in the muscle of the hind-leg. Where $CaCl_2$ had to be injected with the spores, equal volumes of spores and fresh 5 % $CaCl_2$ were mixed and 0·1 ml. injected. Each experiment was concluded on the seventh day. The LD 50 end-point was determined by the method of Reed & Muench (1938), using the data from several consecutive tests.

*Staining methods*

Smears from mouse-lesions were stained by the modified Ziehl-Neelsen method for demonstrating acid-fast spores and also vegetative cells. Histological sections were stained by a combination of the above together with haemalum and Claudius Gram; carbol-fuchsin at 60° C. for 20 min.; wash in water, differentiate in 3 % acid alcohol; wash in water, stain haemalum 15 min.; wash, stain 1 % crystal violet 2 min.; wash, add half saturated aqueous picric acid 1–3 min.; blot, decolorize with 0·1 % picric acid in aniline until no more crystal violet comes out; blot, wash, rapidly dehydrate in absolute alcohol; clear in xylol and mount.

## EXPERIMENTAL RESULTS

### (I) *Injection into mice of spores without adjuvant*

(1) *Intact spores*

Within 2–5 days of being given an injection of spores, the mice died after developing local and general symptoms of botulism. Both intramuscular and intraperitoneal injections gave positive results, the latter route, as is the case with toxin, being slightly the more effective. Several suspensions varying in concentration from 2 to $4 \times 10^8$ per ml. (total count) were used and the volume of the LD 50 was 0·05–0·2 ml.

Table 1 gives the summarized results from the intramuscular injection of mice with three suspensions of strain 4587 and one of strain Hall.

With strain 4587 the number of spores in the mouse LD 50 varied slightly from batch to batch with a mean total count $21{\cdot}2\ (\pm 7{\cdot}3) \times 10^6$. The strain Hall produced a similar fatal toxaemia, but the limited experiments indicated that a still greater number of spores was necessary.

Table 1. *LD*50 *in mice of spores injected intramuscularly without adjuvant*

| | Batch | Ml. | Total count ($\times 10^6$) | Viable count ($\times 10^6$) |
|---|---|---|---|---|
| Strain 4587 | C | 0·055 | 22·7 | 11·6 |
| | D | 0·075 | 23·0 | 9·8 |
| | F | 0·08 | 17·8 | 4·8 |
| | Mean | | 21·2 (± 7·3) | |
| Strain Hall | A | 0·14 | 53·1 | 17·6 |

That the symptoms and death of the mice were associated with the spores present in the injection was shown by the fact that on no occasion did the controls consisting of excess doses of the suspending fluids alone ever produce any effect.

To eliminate the possibility that a fatal amount of toxin remained on the spores despite the original heating at 80° C., the suspension of spores needed for each day's experiments was reheated (80° × 60 min.) before use. This treatment caused no alteration in the effectiveness of a given spore suspension, nor did the pre-treatment of the spores with specific antitoxin, both of which processes were shown completely to detoxify controls consisting of simple solutions of toxin (e.g. 60,000 LD/ml.).

*Histo-pathology* (Pl. 5, figs. 1–3). Examination of the local lesion in the muscle showed that the spores did not germinate, but were to be found—liberally interspersed with polymorphs—in compact foci either within the muscle, or in the connective tissue, and intramuscular spaces. A few polymorphs containing spores had migrated away from the main lesion.

Where the mass of spores and polymorphs lay within the muscle, the fibres of the latter were broken by being forced apart and fragments had become necrotic. The periphery was defined by a cellular zone formed by proliferating sarcolemma nuclei. In connective tissue the circumscribed mass of spores was again interspersed with polymorphs. Some mononuclears occurred nearer the margin which often took the form of an inflammatory tissue reaction. The spores, to be seen in exceedingly large numbers within the foci, were acid-fast when stained except for a very small number which stained with the counterstain as would bacilli. During the examination of the long series of films and sections prepared from mice immediately after their death, no spores were found to have germinated. Only where the post-mortem had been delayed for a few hours were scanty vegetative cells found among the predominating spores.

*Cause of death.* Mice treated prophylactically with type A botulinum toxoid or with Type A antitoxin were completely immune to the spores. This confirmed that death resulted from the specific toxaemia.

The routine pre-treatments of the spores already described having removed the possibility that toxin remained in the supernatant or on the surface of the spores, it appeared that, in the animal tissues, toxin must emerge from the interior of the spores by a simple physical process or as a result of biochemical activity, and experiments were carried out to clarify the position.

(2) *Extraction of spores*

In order to prove or disprove the theory that toxin existed within the spores, attempts were made to extract toxin from the spores under conditions which precluded germination. Full strength stock suspensions ($4 \times 10^8$ per ml.) were incubated at 34° C. in the following fluids in which botulinum toxin could survive but which were unsuitable for the germination of the spores: 1, 5 and 10 % sodium chloride; 1 % sodium phosphate-0·2 % gelatin diluent; and 1 % pepsin (pH 2·0). The extracting fluids were tested for botulinum toxin by the injection of mice with 0·5–1·0 ml. amounts, after periods varying from a few hours up to 6 days. In no instance did the mice die or show symptoms of botulism.

Suspensions of spores and also of vegetative cells were treated with strong solutions of crystalline lysozyme but no lysis resulted. The suspensions agglutinated into floccules and stained more vividly in fixed films, but no alteration in morphology occurred, and the suspending fluid remained innocuous.

(3) *Disintegration of spores*

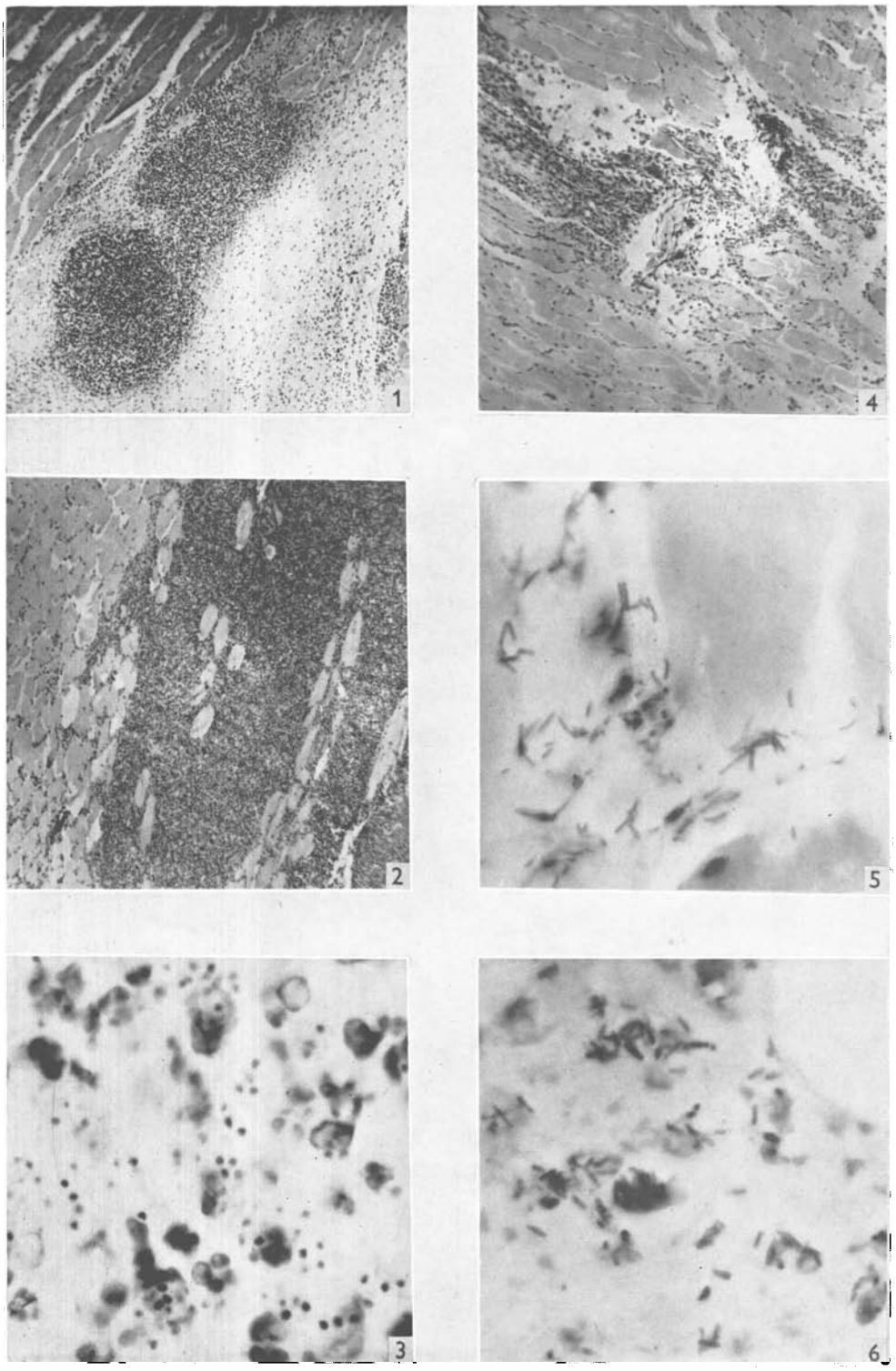
The effect of disintegrating the spores in a Mickle shaking machine was finally tried and the results were positive. Six ml. of heated and antitoxin-treated spores ($5 \times 10^8$ per ml.) were shaken for 15 min. with about 3 ml. ballotini beads (0·2 mm. diameter) at 0–4° C. The concentration of intact spores was reduced to 0–0·1 % of the original, and the shaking had an even more marked effect on the viable count which fell to 0·004 %. A supernatant, free of particulate debris, was obtained by centrifuging at 0–4° C. for 2 hr. at 14,000 rev./min., and this was tested for botulinum toxin. The intraperitoneal injection of the supernatant of the disintegrated suspension produced typical fatal botulism in mice. The LD 50 was 0·05 ml. on one occasion and 0·1 ml. on another (Table 2). The prophylactic use of type A botulinum antitoxin (100 units per mouse) completely protected mice from 0·12 ml. of the toxic product of Exp. 2, which dose killed 8/10 unprotected mice.

Table 2. *The toxicity of disintegrated botulinum spores*

| | Exp. 1 | Exp. 2 |
|---|---|---|
| Original suspension (total count/ml.) | $4{\cdot}6 \times 10^8$ | $5{\cdot}1 \times 10^8$ |
| Disintegrated suspension, LD 50 (ml.) | 0·05 | 0·1 |
| Spores from which LD 50 is released | $2{\cdot}3 \times 10^7$* | $5{\cdot}1 \times 10^7$* |

* This calculated figure may in actuality be smaller if any released toxin has failed to appear in the supernatant because of its destruction during the shaking.

That the toxin was released from within the spores was proved by the fact that the supernatant of the stock spore suspension was innocuous in a volume of 0·5 ml., and the heat and antitoxin treatments individually were capable of destroying a potent concentration of toxin had it been present on the outside of the spores or free in the suspending fluid. Despite the known tendency of botulinum toxin to become adsorbed non-specifically the supernatant, after the shaking treatment and the centrifugation, was 4–5 times more toxic than the resuspended spore-debris. It is most probable that toxin destruction during the 15 min. shaking reduced the

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potency of the products, as the activity of a control solution of botulinum toxin which was shaken in parallel with the spores fell from $1 \times 10^5$ LD/ml. to 25 % of its original value.

The toxin presumably survived the changes in the cell cytoplasm during the formation of the spore. Its resistance to successive exposures of a temperature of 80° C. is interesting, and the discovery of toxin within the spores offers a rational explanation of how an injection of botulinum spores can kill an animal without germination occurring. Presumably, if a large enough mass of spores is present, a fatal dose of toxin is released during their phagocytic digestion.

### (II) *The injection of spores with calcium as adjuvant*

It is well known that calcium salts have an activating effect on the spores of several species of anaerobe when injected into experimental animals before or together with the micro-organisms. The enhancing of the infectivity is greatest when the spores are deposited in the area of tissue devitalized by the calcium salt, and in the present experiments, when mice were injected intramuscularly with mixtures of botulinum spores and 2·5 % $CaCl_2$, a local focus of infection resulted similar to that found in comparable experiments with the gas-gangrene anaerobes.

As was the case with the earlier experiments, controls clearly showed that the symptoms and death of the mice were due to botulism, because mice previously immunized with toxoid or with antitoxin were completely resistant to the injection of spores with calcium. The suspending fluid of the spore suspensions was shown to be free of toxin, and if before being used in an experiment the spores were treated with antitoxin and later washed, no alteration was found in their lethality.

Mice injected with 0·05 ml. of the calcium solution alone showed only slight transient swelling of the leg, but when toxin-free spores were included in the injection, lameness and local paralysis of the leg progressed to fatal general botulism in from 2 to 5 days.

*Histo-pathology* (Pl. 5, figs. 4–6)

Films and sections of the local myositis prepared from moribund or recently dead mice showed the vegetative form of *Cl. botulinum* in varying numbers. Macroscopically the affected leg was swollen with slightly haemorrhagic oedema and the muscle had a zone of congestion around the central core of necrosis resulting from the $CaCl_2$. Microscopic examination showed that the $CaCl_2$ alone produced severe degenerative changes in the muscle with areas of coagulative necrosis, dilation of small blood vessels, oedema and extravasation of blood. The lesion resulting from the LD 50 mixture of spores and $CaCl_2$ closely resembled the above, in addition to which there was a characteristic limiting zone rich in proliferating sarcolemma nuclei. Polymorphs and mononuclear cells were present in small numbers. Acid-fast spores were scanty but prolonged search revealed these and also small but dense groups of vegetative cells. The few nests of proliferating *Cl. botulinum* occurred in the central necrotic zone, in small patches characterized by an accumulation of debris from cells and spores. A minor feature of the lesion was the presence of

faintly stained, non acid-fast, spheroidal bodies which appeared to be the remains of spores damaged by the $CaCl_2$.

(1) *LD 50 of spores plus calcium.* The same stock spore suspensions were used as for the earlier experiments with spores alone, but when $CaCl_2$ was included in the intramuscular injection the LD 50 was much smaller and contained far fewer spores than could cause a fatal toxaemia without germination occurring. The primary factor determining the fatal dose of a mixture of spores and calcium must be the concentration of fully viable spores, whereas the comparable factor in the case of plain spores would be the total amount of toxin available for release from spores whether dead or alive. As the several spore suspensions vary in their proportions of viable spores to total spores so also will the ratio of plain LD/calcium LD vary from batch to batch and among strains according to the viability and toxin content of their component spores. Thus with four batches of spores of strain 4587 the LD 50 of plain spores was respectively 55, 105 108, and 240 times larger in volume than the LD 50 of a mixture with calcium. The mean value of 127 gives some idea of the enhancement of pathogenicity as a result of the calcium allowing germination to occur. Experiments about to be described have shown that all spore suspensions suffer a loss of about 50 % of their concentration on coming into contact with 2·5 % $CaCl_2$, and it therefore follows that the enhancement of pathogenicity, based on the volume of sound spore-suspension constituting the plain and activated LD 50, is actually of the order of 250.

(2) *The effect of* 2·5 % $CaCl_2$ *on botulinum spores.* Using two separate batches of spores of strain 4587 the effect of contact with 2·5 % $CaCl_2$ at room temperature was studied in some detail. In seven instances the sample for counting was withdrawn from the mixture immediately or in a few cases up to 30 min. later. The mean residual viable count as compared with control suspensions in distilled water was $53{\cdot}5 \pm 7{\cdot}5$ %. Comparable data from total counts of the spores surviving contact with the $CaCl_2$ revealed a similar but smaller loss with a mean survival of $65{\cdot}5 \pm 7{\cdot}8$ %. No clear evidence was obtained from the haemocytometer to explain the fate of the missing spores. None were seen in a process of disintegration and there was no visible aggregation of spores or fragments, but there were present a number of particles—not dissimilar to spores in size but having an indistinct and indefinite outline—which might have been spores in the act of lysing. Similar ill-defined spores were also seen in stained sections of lesions from spore plus calcium mixtures.

The above general finding that calcium caused a greater fall in the viable count than in the total count was emphasized when six total and viable counts were made in parallel from one of the suspensions before and after contact with the $CaCl_2$. The residual total counts after the calcium treatment expressed as percentages were 49, 60, 56, 57, 64 and 68 with a mean survival of $59 \pm 6{\cdot}9$. The respective viable percentages were 33, 40, 41, 43, 47 and 54 with a mean of $43 \pm 7{\cdot}4$. Thus the mean percentage survival of viable spores is only 73 % of the value obtained from direct counts, which means that after contact with the calcium solution a proportion of spores become non-viable although retaining their morphology.

From the commencement of the study it was obvious that at least part of the

deleterious effect of the calcium occurred as soon as the spores and the calcium solution came into contact, because the drop in the viable count could be demonstrated if a sample was withdrawn (for the necessary ten-thousand-fold dilution in glass-distilled water) within 30 sec. of the mixture having been made. Some indication of the progress of the changes was obtained in three experiments in which total counts made at the moment of mixing and 1 and 3 hr. later showed mean percentage losses of 25, 31 and 33. Thus 75 % of the loss of morphology had occurred within the short period between mixing and sampling.

In three experiments where viable counts were made immediately and 1½ hr. later, the mean losses were 48 and 59·3 % showing that 81 % of the killing effect of the calcium occurred immediately the spores came into contact with it.

Table 3. *Relative effect on botulinum spores of two grades of calcium chloride, and the nitrate and acetate*

| | Total counts | | Viable counts | |
|---|---|---|---|---|
| | Immediately | 1½ hr. | Immediately | 1½ hr |
| Anhyd. calcium chloride, percentage survival | 57 | 49 | 43 | 33 |
| A.R. calcium chloride, percentage survival | 68 | 64 | 54 | 47 |
| Anhyd. percentage / A.R. percentage | 84 | 77 | 80 | 70 |
| Calcium nitrate, percentage survival | — | 46 | — | 35 |
| Calcium acetate, percentage survival | — | 46 | — | 32 |

In one experiment two brands of calcium chloride were compared, the one being the generally used granular anhydrous form, and the other the 'Analar' hydrated form of a strength equivalent to 2·5 % anhydrous. Table 3 shows that both grades of calcium brought about a reduction in the total counts and in the viable counts. The grade in the anhydrous form caused slightly greater damage, as the final total count, being 49 % of the control, was only 77 % of the 'Analar' calcium sample. Similarly, the final viable count of 33 % found with the anhydrous grade was only 70 % of those surviving contact with the 'Analar' grade for 1½ hr. The pH of the 2·5 % solution of the anhydrous form was 8·2 and that of the A.R. salt was 5·6. The relative importance of the pH was not investigated in detail, but the above grades produced changes of the same magnitude when the pH was adjusted to 7·2.

The effect on botulinum spores of two other calcium salts, namely the nitrate and acetate, was examined *in vitro*. The solutions were equivalent in their calcium content to 2·5 % anhydrous $CaCl_2$ and their pH was adjusted to 7·2. The percentage survival rates of the spores after contact with these solutions are shown in the latter part of Table 3. The nitrate and acetate caused losses similar to those caused by the inferior of the two forms of $CaCl_2$. The latter experiments confirm that the Ca ion is responsible for the damage to botulinum spores revealed in the work with solutions of $CaCl_2$.

(3) *The LD*50 *of spores +calcium in terms of viable spores.* Table 4 summarizes the results of extensive tests in mice with injections of spores plus $CaCl_2$ solution. The spore-counts as listed in the table were made from the aqueous suspensions,

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prior to the addition of the calcium solution and the injection of the mice. The extent of the harmful effect of the calcium on the spores has since been revealed and assessed, and in order to arrive at a more accurate value for the spore-counts constituting the LD50 the mean value of the viable counts from the series of experiments has been amended to allow for the loss which occurs when the calcium solution is added to the spores, prior to the mixture being deposited in the muscle of the mouse.

In the case of strain 4587 the estimated number of viable spores in the mouse LD50 is 49,800 when the simultaneous injection of 2·5 % $CaCl_2$ is used as the activating agent.

Table 4. *The mouse LD50 of spores plus calcium chloride*

| Strain | Batch | Ml. | Total count ($\times 10^4$) | Viable count ($\times 10^4$) |
|---|---|---|---|---|
| 4587 | A | 0·00042 | 18·3 | 8·5 |
| 4587 | B | 0·00053 | 22·0 | 11·2 |
| 4587 | C | 0·0007 | 21·3 | 9·0 |
| 4587 | D | 0·0015 | 33·0 | 8·8 |
| | Mean ($P = 0·95$) | — | — | $9·3 \pm 1·9$ |
| | Mean ($P = 0·95$) | (allowing for the *in vitro* fall in viability to $53·5 \pm 7·5$ %) | | $4·98 \pm 1·2$ |

## DISCUSSION

The experiments described in the earlier part of this paper dismiss as negligible the danger from the ingestion of the spores of *Cl. botulinum* in foods which have become contaminated with them. They therefore support the view that the primary factor giving rise to botulism is the toxin elaborated during the initial period of growth of the contaminant and already present in the spoiled foods at the time of ingestion.

It was found that, by the sensitive intramuscular route, mice succumbed only to an injection of some 20 million spores representing about 0·25 ml. of culture specially enriched to give maximum spore production. Assuming man to be of similar susceptibility to the mouse (which among laboratory animals is relatively susceptible), the body-weight difference introduces a factor of about 3500, and the further large factor reflecting the low sensitivity of the natural oral route as compared with parenteral routes, makes the likelihood of a fatal amount of spores being ingested by man extremely remote.

The discovery of botulinum toxin within the spores after these had been heated to 80° C. is of some theoretical interest as it suggests that toxin derived from the vegetative cell persists in a special form in the spore.

The addition of 2·5 % $CaCl_2$ solution to the spores prior to their injection into mice damaged a proportion of the spores but enabled a relatively small number of the survivors to germinate and produce a fatal anaerobic myositis. It is surprising, therefore, that *Cl. botulinum* has not so far been observed in the bacterial flora of cases of gas gangrene.

SUMMARY

1. The injection of mice with botulinum spores, freed from all traces of superficial toxin, can cause death but only if a very large number is given. This number was found in the case of a highly toxic strain to be of the order of 20 million spores given intramuscularly. This dose was fatal in the absence of spore germination.

2. In human botulism it is concluded that the spores of *Clostridium botulinum* present in spoiled foodstuffs are not of importance compared with the toxin produced by the initial growth of the contaminating organisms.

3. Toxin could be demonstrated in the cytoplasm of botulinum spores when these were disintegrated by mechanical means. This is of theoretical interest as the toxin within the spores is more heat-stable than the extracellular form.

4. A local anaerobic myositis could be produced in mice by the intramuscular injection of spores plus 2·5 % $CaCl_2$. The number of viable spores in the LD 50 was of the order of 50,000.

5. Calcium chloride solution in the commonly used strength of 2·5 % was shown regularly to cause a drop in the number of botulinum spores in a given suspension of some 40 % and a parallel loss of viability of about 60 %.

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EXPLANATION OF PLATE 5

Fig. 1. Deposit of botulinum spores surrounded by polymorphonuclear leucocytes in intermuscular connective tissue. (× 62.)

Fig. 2. Deposit of botulinum spores and polymorphonuclear leucocytes within the muscle. (× 98.)

Fig. 3. Higher magnification of Fig. 1, showing ungerminated botulinum spores. (× 1375.)

Fig. 4. Cellular boundary separating the upper normal muscle from the lower area damaged by $CaCl_2$ plus spores. (× 100.)

Fig. 5. Vegetative *Cl. botulinum* in intramuscular lesion from $CaCl_2$ plus spores. (× 1075.)

Fig. 6. Vegetative *Cl. botulinum* in intermuscular lesion from $CaCl_2$ plus spores. (× 1180.)

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