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The production of *Clostridium botulinum* type A, B and D toxin in rotting carcasses

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SUMMARY

Carrion is a major source of botulinal toxin for animals. A type D strain of *Clostridium botulinum* differed from type A and B strains in producing (1) much higher concentrations of toxin in putrefying mouse carcasses at several different temperatures over a period of 35 days, (2) toxicity that sometimes persisted in mouse carcasses for at least a year, and (3) mouse carcasses with exceptionally high oral toxicity. Fish carcasses were much less favourable than mouse carcasses for type D toxigenesis.

The study, together with earlier studies on types C and E, indicated that carrion contaminated with *C. botulinum* type C or D is likely to be particularly dangerous for animals that may ingest it. This accords with the observation that carrion-transmitted botulism in animals is usually caused by type C or D.

INTRODUCTION

Animals are much more commonly affected than man by botulism, largely because they often eat putrid material, such as carrion.

Clostridium botulinum types A, B and E are the most frequent causes of human botulism and are sometimes responsible for the disease in animals. Types C and D, on the other hand, are notorious for producing botulism in animals but, for reasons that remain unclear, rarely do so in man. Possibly in nature high concentrations of C and D toxin are produced mainly in putrescent material, acceptable to animals as food but repellant to man [1]. Potential interactions between other factors (toxin type, and species variation in susceptibility and in intestinal absorption of toxin) should also be borne in mind [2–4].

C. botulinum is not a normal inhabitant of the gut [5] but is present in the alimentary tract of animals that have recently ingested contaminated material, toxic or otherwise. If such an animal dies, the organism will invade its rotting carcass and produce a large amount of neurotoxin, which is lethal if consumed. Thus carrion is one of the main sources of botulinal toxin for animals.

In experiments on toxigenesis in carrion, Notermans and colleagues [6] found toxicity of $\leqslant 5 \times 10^4$ mouse intraperitoneal LD50/g in the tissues of Pekin ducks dosed orally with *C. botulinum* spores (type B, C or E) before being killed and their carcasses incubated at 25 °C for 3–4 days.

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Smith and colleagues [1, 7, 8] studied toxigenesis in carcasses incubated at different temperatures for different periods. The results were influenced, often in surprising ways, by the nature of the carrion (mammalian, avian, piscine) and its mixed microflora, and by the types of *C. botulinum* examined (C and E). The present communication describes experiments on *C. botulinum* types A, B and D, which complement these earlier studies.

MATERIALS AND METHODS

The methods, which have already been fully described [1, 7, 8], are outlined briefly below, with mention of modifications where appropriate.

Organisms

C. botulinum type A (strain NCTC 7272) and a proteolytic type B strain (NCTC 7273) were both obtained from the National Collection of Type Cultures. *C. botulinum* type D (strain 1873) was supplied by Dr P. Hunter, Veterinary Research Laboratory, Onderstepoort, South Africa.

Animals

Swiss white mice and goldfish, weighing 20–30 g, were used.

Preparation of toxic carcass homogenates

Each animal (mouse or goldfish) used for this purpose was dosed orally with 2000–3000 spores (dose volume 0·25 ml) and killed within a few minutes by cervical dislocation (mice) or a blow to the head (goldfish). Carcasses were wrapped individually in self-seal polythene bags (size 89 × 144 mm, gauge 200; BDH). These bags were enclosed, in groups, in a larger polythene bag and an outer autoclavable bag (Sterilin), both closed with a thick rubber band. The carcasses were then incubated at the temperatures and for the times shown in Tables 1–4. After incubation each putrefying carcass was homogenized in sufficient gelatin phosphate buffer to form a 10% w/v suspension. Each suspension was then either clarified and sterilized by membrane filtration ('filtered' preparation) or merely clarified ('unfiltered' preparation).

Preparation of toxic pure cultures for comparison with carcass homogenates

McCartney bottles (25 ml) containing Cooked Meat Medium (Difco) were seeded with inocula identical with those administered orally to the mice and goldfish, and incubated alongside the carcasses. After incubation each culture supernate was clarified and sterilized by membrane filtration ('filtered').

Abbreviations used

FMH, filtered mouse homogenate; FGH, filtered goldfish homogenate; UMH, unfiltered mouse homogenate; FC, filtered culture.

Toxin assays

FMH, FGH and FC were titrated, without delay, by injecting decimal dilutions (0·5 ml) intraperitoneally into single mice. UMHs were administered orally in decimal dilutions (0·5 ml) to groups of four mice, as well as being assayed

Table 1. *Toxicity of mouse carcasses and cultures inoculated with* C. botulinum *type A and incubated at different temperatures for different periods*

Incubation			Number of carcasses or cultures showing the stated LD (log_{10})* of toxin per g or ml respectively					
Temperature (° C)	Duration (days)	Specimens† ($n = 6$)	0·0 to 2·3	2·3 to 3·3	3·3 to 4·3	4·3 to 5·3	5·3 to 6·3	6·3 to 7·3
37	7	M	—	—	—	3	3	—
		C	—	—	—	—	4	2
	14	M	—	—	3	3	—	—
		C	—	—	—	—	4	2
	35	M	1	3	2	—	—	—
		C	—	—	—	2	2	2
	365	M	6	—	—	—	—	—
		C	4	—	2	—	—	—
30	7	M	—	—	—	4	2	—
		C	—	—	—	2	3	1
	14	M	—	2	3	1	—	—
		C	—	—	—	—	5	1
	35	M	—	1	4	1	—	—
		C	—	—	—	3	3	—
	365	M	6	—	—	—	—	—
		C	2	—	2	2	—	—
23	7	M	—	1	2	3	—	—
		C	—	—	5	1	—	—
	14	M	3	3	—	—	—	—
		C	—	—	—	1	5	—
	35	M	3	3	—	—	—	—
		C	—	—	1	3	2	—
	365	M	6	—	—	—	—	—
		C	6	—	—	—	—	—
16	7	M	6	—	—	—	—	—
		C	1	5	—	—	—	—
	14	M	—	1	5	—	—	—
		C	—	4	1	1	—	—
	35	M	1	4	1	—	—	—
		C	—	1	4	1	—	—

* Results obtained by mouse intraperitoneal titration of FMHs and FCs.
† M, Mouse carcasses; C, cultures.

intraperitoneally as described above. It seems unlikely that infection, as opposed to pure intoxication, affected the results obtained with UMH, but the possibility cannot be excluded entirely. Mice showing signs of rapidly progressing botulism were killed to minimize suffering.

RESULTS

Toxicity of mouse carcasses and pure broth cultures containing C. botulinum *type A, B or D, after incubation at different temperatures for different periods*

The results, as assessed by mouse intraperitoneal titration of FMHs and FCs, are shown in Tables 1–3, and in Figure 1.

After incubation at 37°, 30°, 23° or 16 °C for 7, 14 or 35 days toxin production

Table 2. *Toxicity of mouse carcasses and cultures inoculated with* C. botulinum *type B and incubated at different temperatures for different periods*

Incubation			Number of carcasses or cultures showing the stated LD (log_{10})* of toxin per g or ml respectively					
Temperature (° C)	Duration (days)	Specimens† ($n = 6$)	0·0 to 2·3	2·3 to 3·3	3·3 to 4·3	4·3 to 5·3	5·3 to 6·3	6·3 to 7·3
37	7	M	—	—	—	4	2	—
		C	—	—	—	—	6	—
	14	M	—	—	—	1	5	—
		C	—	—	—	—	6	—
	35	M	2	2	—	2	—	—
		C	—	—	—	1	2	3
	365	M	6	—	—	—	—	—
		C	1	4	1	—	—	—
30	7	M	—	—	1	1	4	—
		C	—	—	—	2	4	—
	14	M	—	—	2	1	3	—
		C	—	—	—	—	6	—
	35	M	—	1	1	1	3	—
		C	—	—	—	—	1	5
	372	M	6	—	—	—	—	—
		C	2	3	1	—	—	—
23	7	M	—	—	2	4	—	—
		C	—	1	4	1	—	—
	14	M	—	—	—	4	2	—
		C	—	—	—	6	—	—
	35	M	—	—	—	3	3	—
		C	—	—	—	—	6	—
	365	M	6	—	—	—	—	—
		C	5	—	1	—	—	—
16	7	M	3	3	—	—	—	—
		C	5	1	—	—	—	—
	14	M	—	—	—	3	3	—
		C	1	4	1	—	—	—
	35	M	—	—	1	5	—	—
		C	—	—	4	2	—	—

* Results obtained by mouse intraperitoneal titration of FMHs and FCs.
† M, Mouse carcasses; C, cultures.

by the type A strain (Table 1) was, despite individual variation between specimens, almost always generally greater in broth cultures than in putrefying carcasses. The same was true of the type B strain (Table 2) at 37° and 30 °C; at 23° and 16 °C, however, toxin was generated more rapidly in carcasses than in cultures. At 37 °C toxin production by the type D strain (Table 3) was greater in cultures than in carcasses; at 30 °C there was little to choose between the two systems; however, at 23° and 16 °C, although not at 9 °C, toxin was generated more rapidly in carcasses than in cultures. At the comparatively low temperature of 9 °C, type D toxin was still produced in both carcasses and cultures, although in concentrations lower than those reached at the higher temperatures.

As is apparent from Figure 1, the estimated mean toxicity titres (at 30°, 23°, and 16 °C, over the first 35 days of incubation) produced in carcasses by the type

Table 3. *Toxicity of mouse carcasses and cultures inoculated with* C. botulinum *type D and incubated at different temperatures for different periods*

Incubation			Number of carcasses or cultures showing the stated LD (log_{10})* of toxin per g or ml respectively					
Temperature (° C)	Duration (days)	Specimens† ($n = 6$)	0·0 to 2·3	2·3 to 3·3	3·3 to 4·3	4·3 to 5·3	5·3 to 6·3	6·3 to 7·3
37	7	M	—	—	1	2	3	—
		C	—	—	—	—	6	—
	14	M	—	—	—	3	3	—
		C	—	—	—	1	5	—
	35	M	—	—	—	4	2	—
		C	—	—	—	—	6	—
	365	M	6	—	—	—	—	—
		C	—	—	6	—	—	—
30	7	M	—	—	—	—	5	1
		C	—	—	—	—	6	—
	14	M	—	—	—	—	4	2
		C	—	—	—	—	5	1
	35	M	—	—	—	2	3	1
		C	—	—	—	—	6	—
	372	M	2	3	1	—	—	—
		C	—	—	1	3	2	—
23	7	M	—	—	—	—	3	3
		C	—	—	—	4	2	—
	14	M	—	—	—	—	5	1
		C	—	—	—	—	4	2
	35	M	—	—	—	1	5	—
		C	—	—	—	1	5	—
	372	M	1	4	—	—	1	—
		C	—	2	—	3	1	—
16	7	M	—	—	—	2	4	—
		C	—	—	—	6	—	—
	14	M	—	—	—	1	5	—
		C	—	—	—	5	1	—
	35	M	—	—	—	3	3	—
		C	—	—	—	—	4	2
9	7	M	—	—	6	—	—	—
		C	—	—	2	4	—	—
	14	M	—	—	6	—	—	—
		C	—	—	—	6	—	—
	35	M	—	—	6	—	—	—
		C	—	—	—	5	1	—

* Results obtained by mouse intraperitoneal titration of FMHs and FCs.
† M, Mouse carcasses; C, cultures.

D strain were greater than those produced by type B, and much greater than those produced by type A. This was also true after 35 (but not 7 or 14) days of incubation at 37 °C. The means of all the titres recorded in Figure 1, expressed in units of 10^6 LD/g, were as follows: type D, 1·9 (range 0·4–6·0); type B, 0·4 (range 0·0006–0·9); type A, 0·1 (range 0·0001–0·6).

After prolonged incubation (365–372 days) at 37°, 30° or 23 °C, carcasses originally seeded with type A or B spores were no longer toxic, unlike a proportion

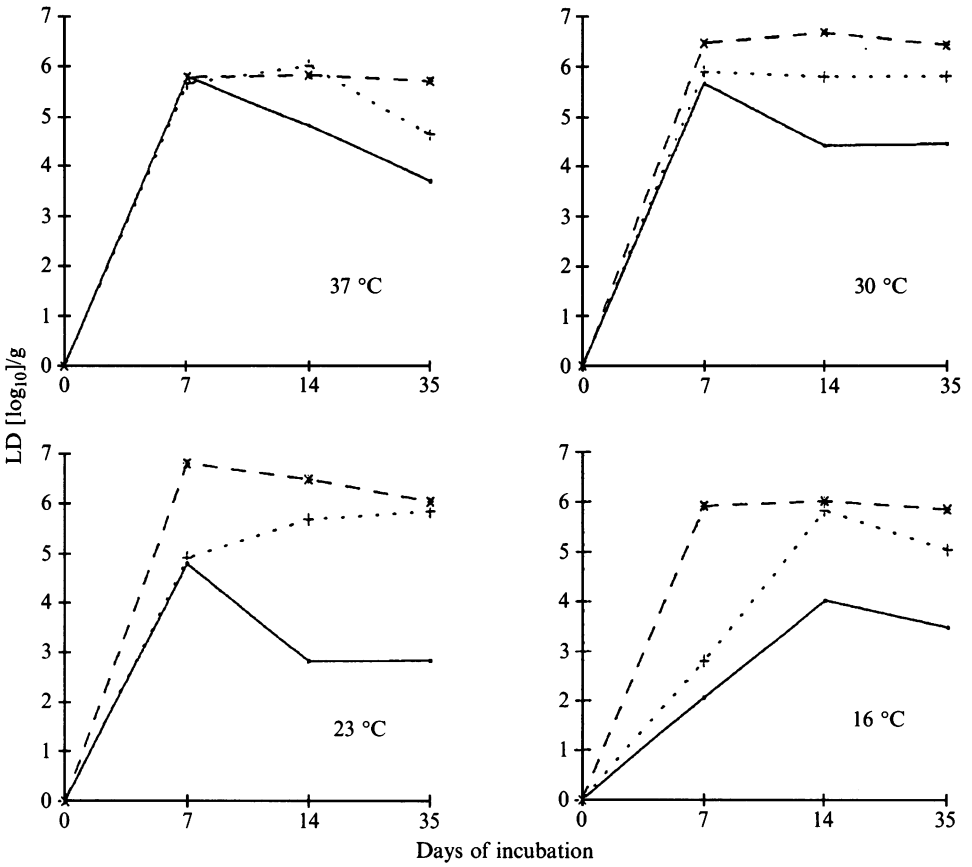

Fig. 1. Toxicity of mouse carcasses containing *C. botulinum* type A, B or D after incubation at one of four temperatures. Each point represents the 'estimated' mean toxin concentration (mouse intraperitoneal LD [$\log_{10}$]/g) of a group of six carcasses. The term 'estimated' is used because the toxin concentration in each individual carcass was assumed to be the mid-point of the range within which the titre fell (see Tables 1–3). ———, Type A; +·····+, type B; *----*, type D.

of the pure cultures. In contrast, some of the carcasses seeded with type D spores still contained toxin after prolonged incubation at 30° and 23 °C, though at concentrations generally lower than in cultures.

Toxicity of fish carcasses and pure broth cultures containing C. botulinum *type D, after incubation at different temperatures for different periods*

The results, as assessed by mouse intraperitoneal titration of FGHs and FCs, after incubation of the carcasses and cultures at 37°, 30°, 23° or 16 °C for 7, 14 or 35 days, are given in Table 4. As to be expected, the toxicity of the pure cultures closely resembled that shown in Table 3. The toxicity of the fish carcasses was much less than that of the cultures, and a comparison of Tables 3 and 4 shows that fish carcasses were much less favourable than mouse carcasses for toxigenesis.

Oral inoculation of mice with UMH containing type A, B or D toxin

Each UMH was prepared from a single mouse carcass, after incubation at 30 °C for 7 days.

Table 4. *Toxicity of goldfish carcasses and cultures inoculated with* C. botulinum *type D and incubated at different temperatures for different periods*

Incubation			Number of carcasses or cultures showing the stated LD (log_{10})* of toxin per g or ml respectively					
Temperature (° C)	Duration (days)	Specimens† ($n = 6$)	0·0 to 2·3	2·3 to 3·3	3·3 to 4·3	4·3 to 5·3	5·3 to 6·3	6·3 to 7·3
37	7	G	—	4	1	1	—	—
		C	—	—	—	—	6	—
	14	G	—	—	2	2	2	—
		C	—	—	—	—	5	1
	35	G	—	3	3	1	—	—
		C	—	—	—	—	5	1
30	7	G	—	—	2	2	2	—
		C	—	—	—	—	6	—
	14	G	—	—	3	2	1	—
		C	—	—	—	—	6	—
	35	G	—	—	4	2	—	—
		C	—	—	—	—	6	—
23	7	G	—	—	1	2	3	—
		C	—	—	—	3	3	—
	14	G	—	4	2	—	—	—
		C	—	—	—	—	5	1
	35	G	3	2	1	—	—	—
		C	—	—	—	—	6	—
16	7	G	—	2	4	—	—	—
		C	—	—	1	5	—	—
	14	G	—	3	2	1	—	—
		C	—	—	—	5	1	—
	35	G	—	6	—	—	—	—
		C	—	—	—	—	4	2

* Results obtained by mouse intraperitoneal titration of FGHs and FCs.
† G, Goldfish carcasses; C, cultures.

Table 5. *Oral inoculation of mice with unfiltered mouse homogenate (UMH) containing type A, B or D toxin*

Type of toxin in UMH	Number (log_{10}) of mouse intraperitoneal LD/g of the tissue from which the UMH was prepared	Number of mice, in groups of four, with fatal botulism after oral administration of mouse tissue diluted 1 in		
		10*	100	1000
A	4·3–5·3	—	—	—
B	5·3–6·3	4	—	—
D	5·3–6·3	4	4	1

* This dilution represents the original (10% w/v) UMH.

UMH containing type D toxin was clearly of much higher oral toxicity than that containing type A or B toxin (Table 5).

In a confirmatory experiment with the type A strain of *C. botulinum*, six UMHs (three 10% w/v and three 20% w/v) were prepared from six mouse carcasses.

Each of these UMHs possessed precisely the same properties as those shown for the type A UMH in Table 5, as regards both (1) the degree of toxicity for mice by the intraperitoneal route of inoculation, and (2) the lack of oral toxicity for a group of four mice.

DISCUSSION

Rotting organic matter, especially carrion, containing *C. botulinum* is the main source of botulinal toxin for animals. Botulism may result from the ingestion of such material itself, or of a foodstuff contaminated by it, or of sarcophagous maggots that have fed on it. Carrion-transmitted type C or D botulism of cattle has been well documented, especially in South Africa, where before the introduction of vaccination *c.* 100000 animals died annually from the disease [9]; in such outbreaks depraved appetite resulting from phosphorus deficiency is a major contributory factor [10]. In recent years the use of poultry litter containing carcass fragments as a pasture fertilizer or foodstuff has led to outbreaks in cattle and sheep [11–13]. So-called forage poisoning in horses and other animals is caused by the accidental incorporation of a small carcass such as that of a cat, rat or bird into hay, silage or other feedstuff, which then becomes contaminated with toxin. Maggots have a role in transmitting toxin from carrion to birds that may eat them, such as waterfowl and pheasants [14].

This paper describes toxigenesis by *C. botulinum* types A, B and D in rotting carcasses and in pure cultures under various conditions of incubation. Because of the arduous nature of the experiments only one strain of each type was examined, a fact that must be borne in mind when attempting to draw conclusions from the study.

A type D strain of *C. botulinum* differed from type A and B strains in producing (1) much higher concentrations of toxin in the carcasses of mice incubated at different temperatures during a period of 35 days, (2) toxicity that sometimes persisted in mouse carcasses for at least a year, and (3) mouse carcasses with exceptionally high oral toxicity. These differences were similar to those noted previously between types C and E [1, 7], the former having much the greater toxigenic potential in mouse carrion.

It was noticeable that the type D strain produced toxic carcasses at a temperature as low as 9 °C, whereas a type C strain [1] failed almost completely to do so at 16 °C. Like type C, but unlike type E [5], type D was much more toxigenic in mouse than in fish carcasses.

The toxicity produced by type A was generally higher in pure cultures than in mouse carcasses under all conditions of incubation. The same was true of types B and D at higher incubation temperatures, but at 23° and 16 °C toxicity was generated more rapidly in carcasses than in cultures, behaviour reminiscent of that shown by types C and E [1, 7].

Although one outbreak of carrion-transmitted botulism in cattle is recorded as having been due to *C. botulinum* type A [15], such outbreaks in animals are usually caused by type C or D [5]. This accords with the present and earlier [1, 7] studies on *C. botulinum* types A, B, C, D and E, which indicate that carrion contaminated with type C or D is likely to be particularly dangerous for animals.

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