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Factors affecting the toxicity of rotting carcasses containing *Clostridium botulinum* type E

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SUMMARY

Mice killed shortly after receiving *c.* 2000 spores of a type E strain of *Clostridium botulinum per os* were incubated at one of five chosen temperatures together with bottles of cooked meat medium seeded with a similar inoculum. After incubation the rotting carcasses were homogenized. Sterile membrane filtrates of the homogenates (10%, w/v) and pure cultures were then titrated for toxicity. Some of the main findings were confirmed with two further type E strains.

Toxicity produced at 37 °C was poor in both carcasses and cultures (200–20000 mouse intraperitoneal LD/g or ml). It was good in both systems at 30 and 23 °C, usually reaching 20000–200000 LD/g or ml, and in carcasses occasionally more; at 30 °C maximal toxicity was reached more quickly in carcasses than in cultures. Prolonged incubation (36–118 days) at 30 or 23 °C resulted in complete loss of toxicity in virtually all carcasses but not in cultures. At 16 °C the development of toxicity in carcasses was strikingly greater than in cultures. At 9 °C neither system produced more than slight toxicity after prolonged incubation. Trypsinization increased the toxicity of cultures but not usually of carcasses. Unfiltered carcass homogenate (10%, w/v) with maximal intraperitoneal toxicity was harmless for mice by mouth in doses of 0·25 ml. These findings differed in important respects from those made earlier with a type C strain.

INTRODUCTION

Types C and D of *Clostridium botulinum* are the more usual causes of botulism in animals but, for reasons that are obscure, rarely if ever affect man. Carrion is a common source of toxin for animals, including birds. Avian botulism, which causes huge losses in waterfowl in lakes and marshes throughout the world, is most often caused by *C. botulinum* type C, but heavy mortality in Common Loons (*Gavia immer*) and gulls (*Larus* spp.) on the shores of Lake Michigan have been attributed to type E (Herman, 1964; Kaufmann & Fay, 1964; Brand *et al.* 1983). *C. botulinum* type E was shown (Notermans, Dufrenne & Kozaki, 1980) to produce toxin in the rotting carcasses of ducks. Type E botulism sometimes produces serious losses in farmed fish, and dead fish undergoing decomposition are a potential source of toxin (Huss & Eskildsen, 1974; Cann & Taylor, 1982; Eklund

et al. 1982). Human type-E botulism is well known to be associated with the consumption of fish or fish products (Smith, 1977).

Smith & Turner (1987) studied the effects of the temperature and duration of incubation on the production of type C toxicity in rotting mouse carcasses and in pure broth cultures. The present report describes similar studies with *C. botulinum* type E.

MATERIALS AND METHODS

The methods, fully described by Smith & Turner (1987), are outlined briefly below.

C. botulinum *spore suspension*

Three type E strains (NCTC nos. 8266, 8550 and 11219) were preserved at −20 °C in plastic bijou bottles containing small volumes of the liquid phase of a sporulated culture in cooked meat medium (Difco).

Preparation of toxic mouse homogenates

Except where stated otherwise, mice were dosed *per os* with *c.* 2000 spores prepared from frozen spore suspension diluted appropriately in distilled water. The animals were killed within 10 min of dosing and sealed individually in plastic bags before being incubated at one of five chosen temperatures. After incubation each putrefying carcass was homogenized in gelatin phosphate buffer to make a 10% w/v suspension. This was then cleared and sterilized by membrane filtration to form 'filtered mouse homogenate' (FMH); or merely passed through muslin to form 'unfiltered mouse homogenate' (UMH). It should be borne in mind that in similar work with a type C strain the 'lethality' of UMH, as demonstrated by the intraperitoneal injection of doubling dilutions into mice, was 2–8 (mean 4) times greater than that of FMH (Smith & Turner, 1987); but filtration reduced the lethality of culture by only 1–2 (mean 1·6) times.

In the first of two experiments with strains 8550 and 11219 the carcass homogenates were prepared as above but from mice inoculated with a 16-day culture in cooked meat medium incubated at 30 °C (dose 0·25 ml of a 10^{-2} dilution, *per os*).

Preparation of toxic pure cultures for comparison with mouse homogenates

Bottles of cooked meat medium (25 ml) were seeded with an inoculum identical with that administered to the mice, and incubated alongside the carcasses. Culture supernate, cleared and sterilized by filtration, was designated 'filtered culture' (FC).

Toxin assay

Toxic preparations from cultures or putrefying carcasses were examined, with the minimum of delay, by injecting decimal dilutions (0·5 ml) into single mice intraperitoneally. Each titration was made in duplicate, one of the two dilution series being made at pH 6·0 in the presence of 0·5% trypsin (Trypsin 1:250; Difco). Both series were allowed to stand for 30 min in a 37 °C incubator before

use. Preliminary tests proved that the toxicity of the spore suspension with which mice were inoculated and cultures seeded was insufficient to affect the titrations, even at the lowest dilution.

RESULTS

Experiments with strain 8266

Tables 1 and 2 show the results of toxicity assays of filtrates (FMH and FC) prepared from mouse carcasses and pure cultures incubated at different temperatures.

Toxicity at 37 °C

The toxicity of carcasses and cultures remained low throughout the 21-day experiment, never rising as high as 2×10^4 LD/g or ml and often being well below (Table 1). The toxicity seen after incubation for 7 days decreased as time progressed. Trypsinization usually increased the toxicity of cultures but had no such effect on carcasses.

Toxicity at 30 °C

The toxicity produced in both systems (Table 1) was much higher (2×10^4 to 2×10^5 LD/g or ml) than at 37 °C. Maximum toxicity occurred within 7 days in carcasses and declined over the next 2 weeks. In cultures toxicity developed more slowly, reaching its maximum after 21 days. Trypsinization increased the toxicity of cultures but not of carcasses. Maximum titres in the two systems were roughly similar.

Toxicity at 23 °C

By the 7th day of incubation the toxicity of carcasses and cultures had reached 2×10^4 to 2×10^5 LD/g or ml (Table 1). By the 14th day some though not all carcasses had become still more toxic, but after a further week there was a suggestion of commencing decline. From the 7th to 21st day the titres of toxin in cultures remained steady and none exceeded 2×10^5 LD/ml. After incubation for 118 days the carcasses had completely lost their toxicity, but cultures were still toxic to some degree. As before, trypsinization increased the toxicity of cultures but not of carcasses.

Toxicity at 16 °C

The toxicity of the carcasses was much greater than that of the cultures throughout this experiment (Table 2). In carcasses toxicity was well developed (2×10^4 to 2×10^5 LD/g) after 7 days' incubation and persisted at this level for at least 2 further weeks. The toxicity of cultures, which was slow to develop, did not exceed 2×10^4 and was often much less. As usual, trypsinization increased the toxicity of cultures, and in this experiment had a similar effect on carcasses after 7, but not after 14 and 21 days' incubation.

Toxicity at 9 °C

Slight toxicity, never more than 2×10^4 LD/g or ml and often much less, developed in carcasses and cultures, but only after prolonged incubation

Table 1. *Toxicity of mouse carcasses and cultures inoculated with* C. botulinum *and incubated at 37, 30 and 23 °C*

Incubation				Number of carcasses or cultures showing the stated LD (log 10) of toxin per g or ml respectively				
Temperature (°C)	Duration (days)	Specimen ($n = 6$)	Trypsinization	0–2·3	2·3–3·3	3·3–4·3	4·3–5·3	5·3–6·3
37	7	M	–	0	4	2	0	0
			+	0	4	2	0	0
		C	–	0	6	0	0	0
			+	0	1	5	0	0
	14	M	–	1	4	1	0	0
			+	4	2	0	0	0
		C	–	3	3	0	0	0
			+	3	3	0	0	0
	21	M	–	5	0	1	0	0
			+	5	1	0	0	0
		C	–	6	0	0	0	0
			+	1	5	0	0	0
30	7	M	–	0	0	0	6	0
			+	0	0	1	5	0
		C	–	0	3	3	0	0
			+	0	0	6	0	0
	14	M	–	0	0	1	4	1
			+	0	0	2	4	0
		C	–	0	4	2	0	0
			+	0	0	5	1	0
	21	M	–	0	1	5	0	0
			+	0	2	4	0	0
		C	–	0	1	5	0	0
			+	0	0	0	6	0
23	7	M	–	0	0	0	6	0
			+	0	0	0	6	0
		C	–	0	1	5	0	0
			+	0	0	0	6	0
	14	M	–	0	0	0	3	3
			+	0	0	0	5	1
		C	–	0	1	5	0	0
			+	0	0	0	6	0
	21	M	–	0	0	1	5	0
			+	0	0	2	4	0
		C	–	0	0	6	0	0
			+	0	0	0	6	0
	118	M	–	6*	0	0	0	0
			+	6*	0	0	0	0
		C	–	0	4	2	0	0
			+	0	0	5	1	0

M, Mouse carcass; C, culture.

* Filtered mouse homogenate (0·5 ml) had lost all toxicity.

Table 2. *Toxicity of mouse carcasses and cultures inoculated with* C. botulinum *and incubated at 16 and 9 °C*

Incubation				Number of carcasses or cultures showing the stated LD (log 10) of toxin per g or ml respectively				
Temperature (°C)	Duration (days)	Specimen ($n = 6$)	Trypsinization	0–2·3	2·3–3·3	3·3–4·3	4·3–5·3	5·3–6·3
16	7	M	−	0	2	4	0	0
			+	0	0	2	4	0
		C	−	6	0	0	0	0
			+	6	0	0	0	0
	14	M	−	0	0	0	6	0
			+	0	0	1	5	0
		C	−	0	6	0	0	0
			+	0	3	3	0	0
	21	M	−	0	0	0	5	1
			+	0	0	1	5	0
		C	−	0	6	0	0	0
			+	0	0	6	0	0
9	7	M	−	6	0	0	0	0
			+	6	0	0	0	0
		C	−	6	0	0	0	0
			+	6	0	0	0	0
	21	M	−	6	0	0	0	0
			+	5	1	0	0	0
		C	−	6	0	0	0	0
			+	5	1	0	0	0
	35	M	−	0	5	1	0	0
			+	1	4	1	0	0
		C	−	6	0	0	0	0
			+	2	4	0	0	0
	125	M	−	2	1	3	0	0
			+	2	2	2	0	0
		C	−	6	0	0	0	0
			+	0	6	0	0	0

M, Mouse carcass; C, culture.

(21–125 days). Once again, trypsinization increased the toxicity of cultures but not carcasses (Table 2).

Administration of unfiltered mouse homogenate per os

UMH was prepared from a carcass which, after incubation for 7 days at 23 °C, contained 2×10^4 to 2×10^5 intraperitoneal LD/g. When given *per os* in a dose of 0·25 ml to six mice it had no effect.

Experiments with strains 8550 and 11219

In the course of two experiments it was found that the concentrations of toxin produced in carcasses incubated at 30 °C for 7 and 10–14 days were similar to those produced by strain 8266. Moreover, as with strain 8266, prolonged

incubation of carcasses resulted in complete loss of toxicity. In the first experiment, each of six carcasses of mice given strain 8550, and five of six given strain 11219, had become non-toxic after incubation at 30 °C for 40–42 days. In the second experiment two further groups of six mice showed complete loss of toxicity after incubation for 36–37 days. Four pure cultures of each strain in cooked meat medium were, however, still toxic after incubation at 30 °C for 130 days; the concentrations of toxin were usually 2000–20000 mouse intraperitoneal LD/ml, and occasionally 200–2000.

DISCUSSION

The main points to emerge from this study of *C. botulinum* type E were as follows. Incubation temperatures of 23 and 30 °C were favourable for the production of toxicity in both rotting carcasses and pure cultures, and the differences between the results obtained with the two systems were comparatively minor; it was noticeable, however, that at 30 °C toxicity was produced more rapidly in carcasses than in cultures. At 16 °C the production of toxicity in carcasses was strikingly greater than in cultures. Temperatures of 9 and 37 °C were unfavourable in both systems for the development of toxicity. Prolonged incubation (36–118 days) at 23 or 30 °C resulted in complete loss of toxicity in virtually all carcasses but not in pure broth cultures. Trypsinization increased the toxicity of cultures but not usually of carcasses. Unfiltered carcass homogenate (10% w/v) with maximal intraperitoneal toxicity was non-toxic for mice by mouth in doses of 0·25 ml.

The findings differed in important respects from those obtained in similar experiments with a single strain of *C. botulinum* type C (Smith & Turner, 1987). As judged by mouse intraperitoneal titration, the type C strain gave rise to maximal levels of toxicity generally much higher than those produced by the three type E strains of the present study. It produced, at 16 °C, little toxicity; at 23 °C, higher toxicity in carcasses than in cultures; at 30 °C, high toxicity in both systems; and at 37 °C, higher toxicity in cultures than in carcasses. As a rule carcasses containing the type C strain did not completely lose their toxicity, even after incubation for 349 days at 30 °C; and unfiltered mouse homogenates given orally to mice produced rapidly fatal botulism.

It is indisputable though mystifying that *C. botulinum* types C and D cause botulism frequently in animals but rarely if ever in man. One possible explanation is that the production of high toxicity by types C and D – but not other types – in natural substrates is strongly favoured by advanced putrefaction. The present study and that of Smith & Turner (1987) give some support to this hypothesis, but much further work is needed. Future studies should include an examination of the behaviour of further type C strains, and strains of types A, B and D in carrion.

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REFERENCES

BRAND, C. J., DUNCAN, R. M., GARROW, S. P., OLSON, D. & SCHUMANN, L. E. (1983). Waterbird mortality from botulism type E in Lake Michigan: an update. *Wilson Bulletin* **95**, 269–275.

CANN, D. C. & TAYLOR, L. Y. (1982). An outbreak of botulism in rainbow trout, *Salmo gairdneri* Richardson, farmed in Britain. *Journal of Fish Diseases* **5**, 393–399.

EKLUND, M. W., PETERSON, M. E., POYSKY, F. T., PECK, L. W. & CONRAD, J. F. (1982). Botulism in juvenile coho salmon (*Oncorhynchus kisutch*) in the United States. *Aquaculture* **27**, 1–11.

HERMAN, C. M. (1964). Significance of bird losses on Lake Michigan during November and December, 1963. *Great Lakes Research Division, Publication No. 11*, University of Michigan, pp. 84–87.

HUSS, H. H. & ESKILDSEN, U. (1974). Botulism in farmed trout caused by *Clostridium botulinum* type E. *Nordisk Veterinärmedicin* **26**, 733–738.

KAUFMANN, O. W. & FAY, L. D. (1964). *Clostridium botulinum* type E toxin in tissues of dead loons and gulls. *Michigan State University Agricultural Experimental Station Quarterly Bulletin* **47**, 236–242.

NOTERMANS, S., DUFRENNE, J. & KOZAKI, S. (1980). Experimental botulism in Pekin ducks. *Avian Diseases* **24**, 658–664.

SMITH, G. R. & TURNER, A. (1987). Factors affecting the toxicity of rotting carcasses containing *Clostridium botulinum* type C. *Epidemiology and Infection* **98**, 345–351.

SMITH, L. DS. (1977). *Botulism: The Organism, Its Toxin, The Disease*. Springfield: Charles C. Thomas.