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Thermal sensitivity of *Clostridium botulinum* type C toxin

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SUMMARY

A sterile suspension containing 950 mouse LD50 per ml of type C botulinum toxin was exposed for various periods to different temperatures. The time required for the 99% (hundred-fold) reduction of toxicity was more than 5 years at −70 °C or −20 °C, 6 months at +5 °C, 3 weeks at +20 °C, 2 weeks at +28 °C, 2 days at +37 °C, 9 h at +42 °C, less than 30 min at +56 °C, less than 20 min at +60 °C, and below 5 min at +80 °C. The results suggest that *Clostridium botulinum* type C toxin, if produced in an ecosystem of the mild climatic zone, might persist there over the winter season and cause the intoxication of vertebrates next early spring in the absence of further microbial toxigenesis.

INTRODUCTION

Type C botulism is a serious disease of certain species of mammals (eg. mink, cattle), poultry and free-living water birds (Roberts *et al.* 1972; Smith, 1976, 1977; Haagsma, 1979; Roberts & Gibson, 1979; Smart *et al.* 1987). In addition to numerous typical outbreaks of avian botulism in warm summer and early autumn seasons, sporadic cases or even small epizootics have been recorded in early spring (Graham *et al.* 1978; Wobeser *et al.* 1983; Rachač, 1986). The origin and source of botulinum toxin in these cases remain obscure. In an attempt to approach this problem, we investigated the thermal resistance of *Clostridium botulinum* type C toxin.

MATERIALS AND METHODS

Preparation of toxic suspension

A sample of the gastrointestinal content with type C botulinum toxin (as determined by toxin neutralization tests using monovalent–ABCDEF–antitoxins) was collected from a wild mallard (*Anas platyrhynchos*) that died in July 1982 during an outbreak of type C botulism among free-living waterbirds in southern Moravia, Czechoslovakia (Hubálek *et al.* 1982, 1984). The homogenate was diluted in cooled physiological saline, cleared by centrifugation for 30 min at 3000 ***g*** and 5 °C, sterile filtered through a 220 nm Millipore membrane, supplemented with gentamicin (200 μg/ml) and distributed in 200 μl aliquots into small polyethylene ampoules which were then leakproof sealed. Final pH of the toxic suspension was 6·5, and the toxin concentration 950 mouse LD50 per ml. Repeated incubations

of the content of several ampoules in meat peptone broth and thioglycollate broth at 37 or 28 °C did not demonstrate any bacterial growth.

Temperature regimes

For various periods of time, the ampoules with toxic suspension were exposed in the dark at different temperatures (with ± limits): −70 ± 5 °C ('Revco' ultra-freezer), −20 ± 3 °C (mechanical freezer), +5 ± 2 °C (refrigerator), +20 ± 2 °C (air-conditioned room), +28 ± 0·5 °C (incubator), +37 ± 0·5 °C (incubator), +42 ± 0·5 °C (incubator), +56 ± 0·3 °C (water bath), +60 ± 0·1 °C (ultrathermostat U2^c MLW Medingen), and +80 ± 0·1 °C (ultrathermostat of the same type).

Toxin assay

Residual toxin concentration in the exposed samples was determined by intraperitoneal inoculation of serial tenfold or fourfold dilutions (in physiological saline) into 25-day old SPF outbred ICR mice (Velaz Praha). Four mice per dilution were each injected with 0·4 ml and observed for 5 days, though specific deaths occurred only within the first 3 days after inoculation. The toxicity was expressed as $\log_{10}$ median mouse lethal doses (LD 50) per ml of the suspension. The initial titre of the toxic suspension was $10^{2·90}$ LD 50/ml, and the rates of toxin inactivation by 90 % (i.e. a decrease by 1·0 log LD 50, tenfold) and 99 % (a 2·0 − log LD 50 decrease, hundredfold) were estimated from graphic plots.

RESULTS

As much as 75 and 6 % of the toxicity still persisted after 5 years of storage at −70 °C and −20 °C, respectively. Table 1 and Fig. 1 show the 90 % (10 % residual toxicity) and 99 % (1 % residual toxicity) inactivation rates of *C. botulinum* type C toxin at different temperatures. No toxin was detectable, when using an initial concentration of 950 LD 50/ml, after 8 months at 5 °C, 5 weeks at 20 °C, 3 weeks at 28 °C, 4 days at 37 °C, 12 h at 42 °C, 30 min at 56 °C, 20 min at 60 °C, and 5 min at 80 °C.

The regression of log time (hours) necessary to achieve 90 or 99 % inactivation of the toxin against temperature is essentially linear (Fig. 1), and can be expressed by the equations: $Y_{90} = 3·275 - 0·0595X$ (correlation $r = -0·994$), and $Y_{99} = 4·053 - 0·0678X$ (correlation $r = -0·978$), where Y_{90} and Y_{99} are the times (in $\log_{10}$ hours) required for the inactivation of type C botulinum toxin by 90 % and 99 % respectively, and X is temperature in °C.

According to these regression equations, the time to 90 % (or 99 %) toxin denaturation might be estimated for particular temperatures as: −70 °C, 3180 (73 365) years; −20 °C, 40 months (30 years); 0 °C, 78 days (16 months); 5 °C, 40 days (7 months); 20 °C, 5 days (21 days); 42 °C, 6 h (16 h); 60 °C, 30 min (60 min); 80 °C, 2 min (3 min); 90 °C, 30 s (32 s); 100 °C, 8 s (8 s).

Except for the higher temperatures, the correlation with the experimental data is good.

Table 1. *Inactivation rates of* C. botulinum *type C toxin at different temperatures*

Temperature (° C)	Time required for inactivation by 90 %	99 %
−70	> 5 years	> 5 years
−20	4 years	> 5 years
+ 5	30 days	6 months
+20	3 days	21 days
+28	2 days	14 days
+37	1 day	2 days
+42	5 h	9 h
+56	< 30 min	< 30 min
+60	< 20 min	< 20 min
+80	< 5 min	< 5 min

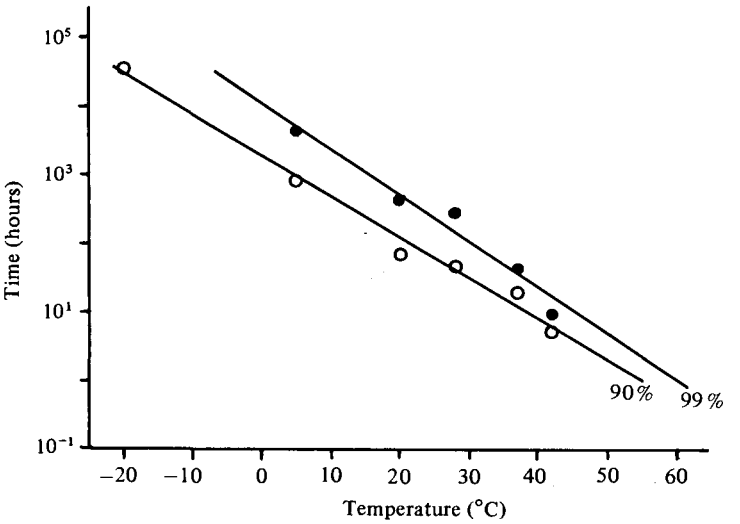

Fig. 1. Regression of time to 90 and 99 % toxin inactivation on temperature.

DISCUSSION

Surprisingly there are few published reports on thermal stability of *C. botulinum* type C toxin. Boroff & DasGupta (1971) mentioned its relative stability at temperatures of 20 °C (*c.* 7 days) or less, but noted rapid (within-seconds) destruction at 80 °C and 90 °C. Residual toxicity of 10 and 1 % were detected after exposure for 2 min at 70 and 80 °C respectively (Prévot & Brygoo, 1953). Yamakawa, Nishida & Nakamura (1983) observed the resistance of type C toxin to an exposure of 10 min at 50 °C, while complete inactivation occurs after 10 min at 60 °C. Smart & Rush (1987) described a 99 % destruction of type C toxin in 5–15 min at 60 °C at pH 7, but only in 15–60 min at 70 °C at pH 5. The effects of pH, ionic strength, presence of proteins and other compounds on the heat stability of botulinum toxins are well documented (Scott & Stewart, 1950; Roberts & Gibson, 1979; Smart & Rush, 1987).

In our study, the major experimental conditions were a pH of 6·5, the absence of access to free air, constant temperature environment, and the absence of viable

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bacteria. Our results also demonstrated a high sensitivity of type C toxin to temperatures above 55 °C. Even at avian body temperature (42 °C) a hundred-fold decrease in toxicity took place within 9 h. The inactivation rate at 37 °C was also high. However, prolonged persistence of the toxicity was found at temperatures below 30 °C, and particularly long-term persistence at temperatures below 10 °C. For instance, the time required for the 99% inactivation of toxicity at 5 °C is 6 months. This could enable 'overwintering' of type C botulinum toxin, if it is produced in a sufficient quantity during warm weather within a generally mild climatic zone. Graham *et al.* (1978) demonstrated experimentally that the toxin (initial concentration – 10^5 mouse MLD/ml) might well be stable over 10 months in semi-natural circumstances.

The exact source of avian intoxication in the spring cases of botulism remains to be determined. However, it seems to be associated with the bottom habitat (benthos, dead invertebrates, rotting carcasses) in lakes and ponds, since the ducks affected in the spring are largely diving species like *Aythya affinis* in Canada (Wobeser *et al.* 1983) or *Aythya fuligula* in Czechoslovakia (Rachač, 1986). High levels of type C toxin (up to 10^6 LD50/g) have been detected by many workers in sarcophagous maggots collected from carrion (Haagsma *et al.* 1972; Duncan & Jensen, 1976; Wobeser *et al.* 1983; Shayegani, Stone & Hannett, 1984; Grüll, Bauer & Sagmeister, 1987), and less frequently in other invertebrates, beetle larvae (Duncan & Jensen, 1976) or chironomid larvae (Grüll, Bauer & Sagmeister, 1987). Interestingly, the prototype strain of *C. botulinum* type C was isolated from maggots of the fly *Lucilia caesar* (Bengtson, 1922).

Toxin production in early spring is very unlikely to occur in natural ecosystems in mild climatic zones since the minimum temperatures for toxigenesis in *C. botulinum* type C are reported as *c.* 16 to 20 °C (Segner, Schmidt & Boltz, 1971; Haagsma, 1979; Smith & Turner, 1987).

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