

STUDIES IN THE PHYSIOLOGY OF *CLOSTRIDIUM BOTULINUM* TYPE E

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[Manuscript received July 26, 1956]

Summary

Ten strains of *Clostridium botulinum* type E have been studied at 12 temperatures between 2.5 and 45°C. Growth proceeded consistently from spore inocula at temperatures between 5 and 37.5°C, but some strains developed slightly at temperatures up to 45°C. Maximum rates of growth occurred at 35°C. The upper and lower temperature limits were several degrees lower than for type A and B strains.

The heat resistance of the spores was about 1/1000th that of spores of some type A and B strains. Type E toxin was found in greatest amount in cultures grown at 25°C. The toxin was most stable at pH 4.5-5 but more heat labile than type A toxin.

Two of 22 samples of marine mud yielded cultures containing *botulinum* toxin. The possible marine origin of type E strains is discussed.

I. INTRODUCTION

Although the physiology of *Clostridium botulinum* has been studied extensively, most of the work has been done with strains of types A and B. Relatively little attention has been given to type E strains which, although not as common as types A and B, have, nevertheless, caused a number of fatal cases of human botulism. The epidemiology of type E botulism has been reviewed by Dolman and Chang (1953) who have shown that, with one exception, all cases were associated with fish or marine mammals. The present paper reports some physiological studies made on 10 type E strains and shows that they constitute a homogeneous group differing in several respects from strains of types A and B.

II. METHODS

The techniques and media (neopeptone-yeast extract-glucose (N.Y.G.)) used were the same as those described by Ohye and Scott (1953). The organisms were grown in evacuated sealed tubes and growth was measured nephelometrically. Some experiments were also made in cooked meat medium (C.M.M.). In view of the much lower heat resistance of type E spores possible non-sporing contaminants were eliminated by heating the sealed tubes for 15 min at 60°C rather than 10 min at 80°C. Each μA of the nephelometer reading was equivalent to approximately $5.5 \pm 0.3 \times 10^{-2} \mu\text{g}$ dry weight or $5.1 \pm 0.55 \times 10^4$ cells. According to these values, the type E cells have about three times the light scattering capacity of cells of types A and B, but this may be due to short chains of the type E organisms being counted incorrectly as single cells.

Heat resistance of the spores was determined by the methods of Olsen and Scott (1950) using tubes of pork infusion thioglycollate starch agar incubated for 2 weeks at 25°C. The heat stability of the toxin was determined by methods described by Scott and Stewart (1950).

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The 10 strains used were numbered 101 to 110 in this Laboratory. Strains 101 and 102 were isolated by Dr. Elizabeth Hazen, 101 (35396) from canned sprats, and 102 (36208) from smoked salmon (Hazen 1937, 1938). Strains 103 and 104 were isolated by Prof. C. E. Dolman, being respectively his Nanaimo (Dolman and Kerr 1947) and Vancouver herring strains (Dolman *et al.* 1950). Strains 105–110 were originally isolated in U.S.S.R. and two of them were described by Gunnison, Cummings, and Meyer (1936). These were received as strains 7–4, 2, 8, 151, 30–6, and 30–17 respectively. Strain 102 was obtained from Dr. Hazen, strains 103 and 104 from Prof. Dolman, and the remaining 7 strains from Prof. K. F. Meyer.

III. RESULTS

(a) Temperature Relations

(i) *Rates of Growth.*—Although all 10 strains were studied at 12 temperatures from 2.5 to 45°C complete results were obtained for only nine strains. Spores of strain

TABLE 1
MEAN RATES OF GROWTH AND THEIR STANDARD ERROR AT VARIOUS TEMPERATURES IN N.Y.G. MEDIUM

Temperature (°C)	No. of Strains	Mean Rate (divisions/hr)	S.E. of Mean
5.0	9	0.0234	0.0019
10.0	9	0.139	0.0084
15.0	10	0.326	0.0078
20.0	9	0.539	0.0198
25.0	10	1.006	0.0251
30.0	9	1.492	0.0379
35.0	10	1.777	0.0497
37.5	9	1.456	0.1221
40.0	9	1.196	0.2307

101 often failed to initiate growth, and when growth of this strain was observed the lag period was usually much greater than for the other strains. Although the observed rates of growth of strain 101 were very similar to the other strains at several temperatures, there is no certainty that it does not differ from the others, and subsequent discussion will, therefore, be limited to the remaining nine cultures.

In N.Y.G. medium all strains developed from a spore inoculum at temperatures between 10 and 37.5°C. Growth at 37.5°C was often delayed for 1–3 days. At 5°C only six strains grew from a spore inoculum, but all strains grew when growth was initiated at 10°C. Similarly at 40°C only three strains developed from a spore inoculum, whereas all strains grew when growth commenced at 30°C. The mean rates of growth and their standard errors are given in Table 1, the data for 5, 37.5, and 40°C being based on cultures transferred from intermediate temperatures. Variability of the data, expressed as a percentage of the mean, is least at temperatures between 15 and 35°C.

The mean rates of growth and their temperature coefficient are shown also in Figure 1 together with the rates previously reported for types A and B strains. For the type E strains the maximum rate of growth occurs at *c.* 35°C compared with

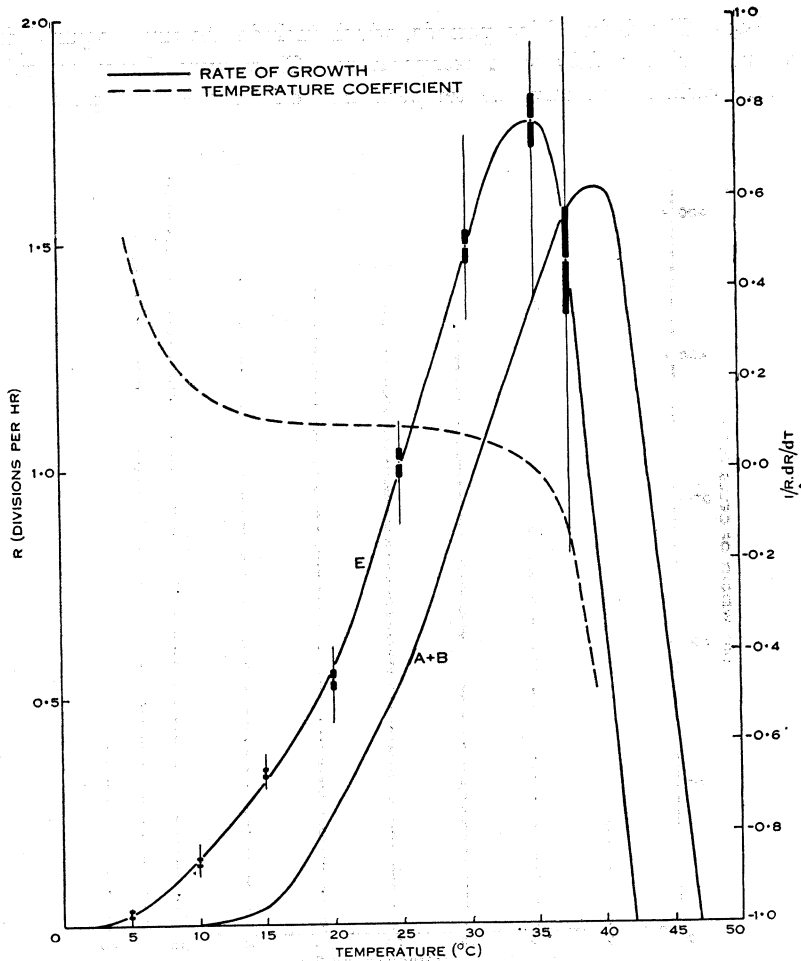


Fig. 1.—Relation between the mean rate of growth (R), the temperature coefficient of R , and the temperature (t) in N.Y.G. medium. The heavy vertical lines show the mean value of $R \pm$ its standard error; the light vertical lines show the range of values observed at each temperature. The curve A and B shows the mean values of R previously obtained for types A and B strains (Ohye and Scott 1953).

40°C for A and B strains. It will be noted that the curves intersect at about 37°C; and so result in a fortuitous equality of growth rates at this temperature. It is clear that the type E strains have minimum temperatures for growth 8–10°C below the minimum for types A and B and that the maximum temperatures are also about 5°C less. At several intermediate temperatures a temperature difference of 5–7°C suffices to equalize the rates for types A, B, and E strains.

As shown previously for types A and B strains, the temperature coefficient for type E strains also becomes large at low temperatures. The rates of growth are changed by about 10 per cent. per °C at 25°C, by 20 per cent. at 10°C, and by 50 per cent. at 5°C.

(ii) *Lag*.—The deduced lag periods, which include the time required for spore germination, varied widely with temperature. They were, however, subject to considerable variation at any one temperature and for the most part, differences

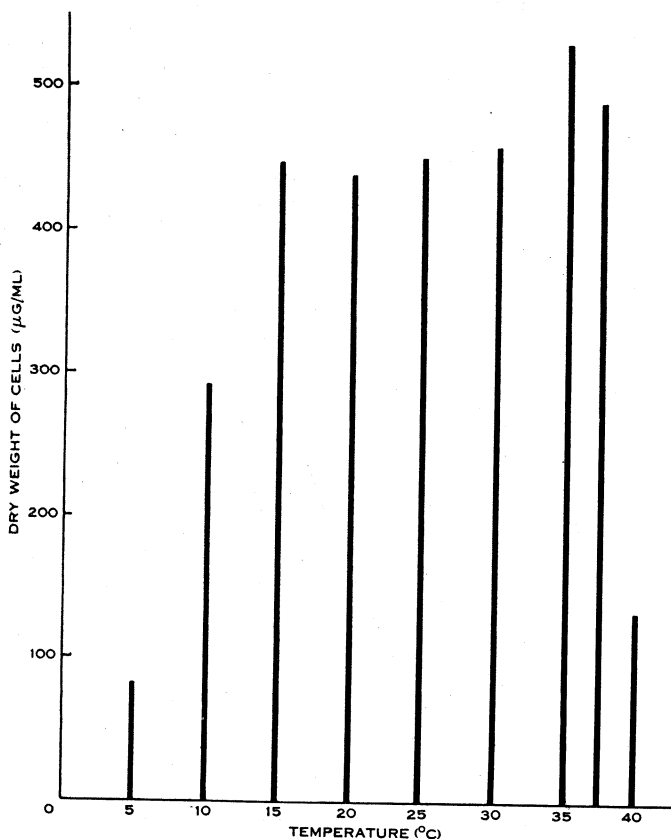


Fig. 2.—Relation between the yield of cells and temperature (N.Y.G. medium).

between strains were not consistent. From 20 to 35°C the mean lag period was about 4 hr with variations from 1 to 10 hr. There were no consistent differences between temperatures within this range. At 10 and 15°C the lag period fluctuated between 9 and 33 hr, the mean value being about 24 hr. At 37.5°C the lag varied greatly between 1–2 hr and 2–3 days.

(iii) *Yield of Cells*.—The maximum yield of cells showed a considerable dependence on temperature as has been reported previously for types A and B strains. The average results for all experiments are shown in Figure 2. The maximum density of the cultures did not vary greatly between 15 and 37.5°C but diminished greatly at temperatures outside this range.

(iv) *Lower Temperature Limits.*—Although only six strains grew from spore inocula in N.Y.G. medium at 5°C all strains grew in C.M.M. at the same temperature. Gas production, sufficient to lift the cooked meat, was evident 3–4 weeks after inoculation. When C.M.M. cultures were opened after 8 weeks at 5°C, toxin lethal to mice was found in four cultures, the titre being of the order of 10 mouse-doses per ml. The remaining five cultures did not kill mice after the injection of 0.2 ml, but some of these presumably contained a little toxin as the mice showed transient symptoms of botulism from which they recovered.

Duplicate tubes of all 10 strains were incubated in N.Y.G. medium at 2.5°C for 22 weeks. There was no change in turbidity, and after heating at 60°C for 30 min the

TABLE 2
VIABLE COUNTS AND pH VALUES OF C.M.M. CULTURES HELD AT 2.5°C FOR 20 WEEKS AND AT 45°C FOR 1 WEEK

Strain	2.5°C			45°C		
	pH	Viable Count/Ml		pH	Viable Count/Ml	
		Unheated	Heated*		Unheated	Heated*
101	6.78	830	570			
102	6.76	<10	<10	6.51	5000	<1
103	6.75	550	700	6.70	7000	<1
104	6.76	900	650	7.17	1120	350
105	6.85	630	820	7.20	830	800
106	6.80	770	930	7.22	940	800
107	6.76	840	650	7.22	540	500
108	6.86	430	500	7.24	820	960
109	6.80	<10	<10	7.20	500	<1
110	6.76	650	600	7.20	170	<1

* Inoculum heated for 15 min at 60°C.

sealed tubes were incubated at 25°C. Growth occurred in all tubes within 24 hr. There is evidence, therefore, that the spores did not germinate and that the medium remained suitable for growth. Similar experiments were carried out in sealed tubes of C.M.M. incubated at 2.5°C for 20 weeks. At the end of this time all tubes were opened. There was no macroscopic or microscopic evidence of growth and when 0.4-ml aliquots of each tube were injected into mice, there were no deaths and none of the mice showed symptoms of botulism during observation for 3 days. Portions of the fluid were taken and 0.1-ml aliquots inoculated, unheated and heated, into tubes of pork infusion thioglycollate starch agar. Results are shown in Table 2. The remaining portion of each C.M.M. tube was transferred to 25°C and growth occurred in all tubes within 24 hr. For seven of the strains, therefore, the evidence is that the spores did not germinate within 5 months at 2.5°C, but for strains 102 and 109, the original inoculum had become largely non-viable.

(v) *Upper Temperature Limits.*—In three series of experiments, only strains 103, 104, and 110 developed in N.Y.G. medium within 5–7 days at 40°C. Strain 103 grew on two occasions and 109 and 110 on only one occasion. In all tubes in which growth did not occur the inoculum remained stable to 15 min at 60°C and growth ensued after transfer to 25°C. When N.Y.G. cultures were transferred from 30 to 40°C all strains grew, but the maximum turbidity for strains 103 and 104 was much greater than for the other seven strains. When N.Y.G. cultures were transferred from 30 to 42.5°C sustained growth occurred only with strains 102, 103, and 104. For the other six strains there was only a small and transient rise in turbidity which at no time was more than four times the turbidity at the time of transfer. When transferred from 30°C the cultures had turbidities equivalent to cell populations of $1-5 \times 10^6$ /ml.

When C.M.M. cultures were inoculated with spores and held for 7 days at 45°C, slight growth occurred with strains 102 and 103 and the inoculum of 109 and 110 became heat labile. With strain 104, about 70 per cent. of the spores became heat labile. With the other strains the spores did not germinate, as judged by viable counts (Table 2) on heated and unheated aliquots of the cultures. Cultures of 102 and 103 showed microscopic evidence of slight growth, a few irregularly stained rods being found in smears. No organisms were seen in stained smears from the other cultures. None of the cultures contained demonstrable amounts of toxin when 0.4-ml aliquots were injected into mice.

The results indicate that growth is unlikely to proceed at temperatures greater than 45°C and that about half the strains are unable to grow at this temperature. There is evidence of some consistent differences between strains, and strains 102, 103, 104, 109, and 110 appear to have maximum temperatures slightly greater than strains 105, 106, 107, and 108.

(b) *Sporulation and Heat Resistance of Spores*

Sporulation varied greatly with temperature and the medium in which the organisms were grown. At 25 and 30°C the yield of spores was much greater than at 37 and 20°C. A number of media including N.Y.G., peptic and papain digests of ox heart, and casitone and peptone solutions, each containing 0.01 per cent. sodium thioglycollate, were tested with and without meat particles at pH levels of 6.0, 7.5, and 9.0. Sporulation was enhanced by the presence of meat particles in all media, the best yields being obtained in papain-digest media with meat particles and an initial pH of 7.5. In this medium as many as 90–95 per cent. of the cells formed spores in 5 days at 30°C. The addition of 0.5 per cent. glucose to this medium greatly increased the yield of cells, but seriously depressed sporulation.

In contrast to types A and B cultures in which motility was readily observed only in young cultures, active motility was well retained in old type E cultures. It was, in fact, common to observe active motility in cells which had already formed a mature, refractile sporangium.

Although it is generally agreed that type E spores are much more heat labile than spores of types A and B strains, quantitative data on rates of destruction of type E spores have not yet been published.

Spores of strains 103 and 108 were heated in M/15 phosphate buffer pH 7.0 and the viable spores enumerated after various periods of heating at 70, 75, and 80°C. The logarithm of the number of viable spores was approximately a linear function of time at all three temperatures and the following decimal reduction times indicate the slopes of the curves. For strain 103 these were 36, 10, and 3.3 min and, for strain 108, 7.8, 3.1, and 0.4 min at 70, 75, and 80°C respectively. The temperature coefficient is of the same order as for data on types A and B spores. The results show that strain 103 is somewhat more heat resistant than strain 108, but illustrate clearly the very low resistance in comparison with types A and B strains.

For strain 103, for instance, a Q_{10} of 10 indicates a decimal reduction time of 2 sec at 100°C. For some type A and B strains, the data of Olsen and Scott (1950) show these to be over 1000 times more resistant at 100°C. As would be expected from the above results the viable counts of type E spores were virtually unaffected by heating for 15–30 min at 60°C.

(c) Toxin Production and Heat Resistance of the Toxin

Mice injected with type E toxin showed, relative to others receiving type A or B toxins, certain differences in the toxic syndrome. While the symptoms of respiratory distress and paralysis were rather similar they had a more rapid onset with type E toxin. Mice receiving more than 10 lethal doses usually showed the first symptoms within 2–4 hr and, indeed, death often occurred within 4 hr. It was unusual for a mouse to die if the initial symptoms were not apparent 6 hr after injection. Almost all mice receiving a lethal dose of type E toxin died within 24 hr of injection, whereas with A and B toxins deaths 2–4 days after injection were not uncommon. Mice showing symptoms of botulism 24 hr after injection of type E toxin almost always recovered and symptoms disappeared within a further 24 hr. Therefore, mice were not observed for more than 3 days after injection.

Several strains produced toxin in C.M.M. within 7 days at 25°C with titres of 1000–5000 mouse-doses per ml. At 30 and 37°C titres were lower and, on occasions, cultures grown at 37°C were non-toxic. In contrast to the effect with type A cultures, the addition of 0.5 per cent. glucose did not enhance the titre, but rather tended to reduce it.

Attempts to concentrate the toxin by acid precipitation were unsuccessful. When C.M.M. cultures of strain 101 were brought to pH 5 with HCl a precipitate formed, but when this was redissolved in phosphate buffer of pH 7 the fluid was no more toxic than the original fluid. Further reduction of the pH to 3.5, 2.5, and 1.5 caused no reduction in the titre.

The heat stability of toxin produced by strain 101 has been examined in several experiments. The toxin is appreciably less resistant than type A toxin, but as with the latter, has its greatest stability at pH 4.5–5. Some typical results are shown in Table 3, in which it may be seen that toxin was destroyed within 5 min at 60°C at pH 7.5 and 3.5 but persisted with a titre of 4^{-2} after 40 min at pH 4.6 and 4.9. Other experiments also showed greatest stability at pH 4.5–5 when toxin was heated at 65°C, the rate of destruction being 6–10 times greater at the higher temperature.

Attempts to increase the heat stability of type E toxin by heating in solutions of high ionic strength were also unsuccessful. When an equal volume of a solution of mixed electrolytes of ionic strength 2.0 (Scott 1950) was added to toxic fluid from a C.M.M. culture and heated at 60°C at pH 5.0 the stability was not increased in

TABLE 3
DESTRUCTION OF TYPE E (101) TOXIN IN C.M.M. FLUID AT 60°C

The results represent the highest dilution to cause death of mice injected with 0.2 ml. Initial titre 4^{-5} . 0 = no deaths with undiluted toxin, — = not done

pH	Time of Heating (min)			
	5	10	20	40
7.5	0	—	—	—
6.3	4^{-1}	4^{-1}	4^0	—
5.5	—	4^{-2}	4^0	—
5.2	—	4^{-1}	4^0	4^0
4.9	—	4^{-3}	4^{-3}	4^{-2}
4.6	—	4^{-4}	4^{-2}	4^{-2}
4.3	—	4^{-4}	4^{-2}	4^{-1}
4.0	—	4^{-2}	4^0	4^0
3.5	0	—	—	—

comparison with another aliquot mixed with an equal volume of distilled water. In this respect, therefore, type E toxin differs from type A toxin for which heat stability was considerably enhanced in solutions of high ionic strength (Scott 1950).

(d) *Cl. botulinum* in Marine Muds

In view of the lower temperature range for the growth of type E strains it was decided to examine some marine muds for the presence of this organism. Twenty-two samples were collected from Tasmanian and New South Wales coastal waters, generally just a few feet below the low tide level. Samples of 10 g were added to 100 ml of C.M.M., heated at 60°C for 30 min, and incubated at 10°C. Good growth of a variety of *Clostridium* spp. ensued in all cultures, and several of the isolates were found to produce spores which were, like type E spores, readily destroyed at 70 and 75°C. After incubation for 30 days aliquots of 0.2 ml were injected into mice. *Cl. botulinum* toxin was detected in cultures from two muds, both collected at 6–8 fm a few miles from Hobart, Tas. These observations were confirmed in cultures prepared from further aliquots of these muds which had been held at 0°C. When the toxic fluid was mixed with antitoxin 1 hr before injection, the mice were protected by type B, but not by types A or E antitoxins. Repeated efforts to isolate the toxigenic organism, and indeed to prepare toxic subcultures, were uniformly unsuccessful. The indication that type B toxin was present in these cultures was unexpected as Ohye and Scott (1953) had found a number of type B strains were unable to grow at 10°C.

Since these experiments were completed, Pederson (1953) reported an outbreak of type E botulism in Denmark in which marinated herring was the causal food. The initial finding of the State Serum Institute was that the herring contained *botulinum* toxin type B. Subsequently, a pure culture of type E *Cl. botulinum* was isolated, the toxin of which was partially neutralized by type B antitoxin, mice receiving type B antitoxin showing much lower mortality. In the light of these results, therefore, it is still possible that the toxin found in cultures from the two marine muds was, in fact, type E.

Pederson (1955) isolated *Cl. botulinum* type E from 16 out of 19 samples of mud collected from Copenhagen harbour, and from Agluitsk Bay, Greenland, but from only one of 19 soil samples collected around the Copenhagen fish markets. These results indicate the prevalence of type E organisms in marine muds.

IV. DISCUSSION

The main feature of the results is the clear demonstration that the temperature relations of type E strains differ substantially from those for the toxigenically related types A and B strains. As the temperature range of microorganisms is an attribute with great genetic stability there is a strong suggestion that the natural habitat of type E organisms is at a lower average temperature than the natural habitat of A and B strains. The isolation of type E organisms from marine muds by Pederson (1955), together with the characteristic association of type E botulism with marine products, suggests a marine rather than a terrestrial habitat.

The finding that many of the clostridia in marine muds produce spores of low resistance to heat shows that the widespread assumption that bacterial spores survive 10 min at 80°C is unsound. Failure to recognize this is probably the reason why organisms producing heat labile spores have not been reported more frequently. For spores like those of strain 108, 10 min at 80°C would suffice to reduce the number of viable spores by a factor of 10^{25} .

There is general agreement that although type E strains grow readily at 37°C the cultures grown at this temperature are frequently non-toxic. It will be seen from Figure 1 that at 37°C the temperature coefficient is already negative and it would not be surprising if some of the normal properties of the organisms are impaired at this temperature. It is also possible that variations in the temperatures actually realized in incubators nominally operating at 37°C could account for some of the differences between observers (Dolman *et al.* 1950; Barron and Reed 1954; Pederson 1955).

It is to be noted that the strains examined in these experiments have been isolated in Europe and various parts of North America, and that, at least in respect of temperature requirements, they constitute a reasonably uniform group. It is true that some of the strains were consistently somewhat more vigorous in growth at temperatures above 37°C, but when compared with the differences from A and B strains these variations within the type E group were relatively small. Although the 10 strains used in the present study include most of those isolated prior to 1955, further observations on additional strains would help in assessing the variations which may normally occur within the type E group.

V. ACKNOWLEDGMENTS

The authors are indebted to Messrs. A. M. Olsen and E. J. F. Wood, Division of Fisheries, C.S.I.R.O., for the collection of mud samples.

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