Effect of Nutrients on Physiological Properties of Clostridium botulinum Type E

B. GULLMAR and N. MOLIN

Swedish Institute for Food Preservation Research (SIK), Göteborg, Sweden

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Eight strains of Clostridium botulinum type E out of twelve tested showed good growth and normal cell morphology in a synthetic medium containing choline. Growth and toxin production by a representative strain was not influenced by repeated subculturing. In the chemically defined medium, acetylcholine, N,N-di-methylethanolamine, and lecinthin could replace choline to get normal cell division and cell morphology of C. botulinum type E. Choline could not be replaced by ethanolamine, N-methylethanolamine, or betaine. A toxigenic strain of C. botulinum type E showed proteolytic, lipolytic, and lecinthinase activity in complex media but not in a synthetic medium. On prolonged incubation in the high temperature range of growth, the toxicity of the culture filtrate decreased in a complex, but not in a synthetic medium. The implications of these findings are discussed.

The uneven distribution of Clostridium botulinum type E spores in different geographical areas (5, 7, 9, 10, 12, 13, 16, 26, 27, 28, 34) and the observation that various fish products have different capacities to promote growth and toxin production (1, 6, 8, 35, 42) have prompted investigations into the behavior of this organism in different chemical and physical environments (2, 36, 38). Lack of suitable chemically defined media has hitherto delayed the elucidation of the nutritional requirements of the organism. Recently, however, a few reports describing growth and toxin production of C. botulinum type E in chemically defined media have been published (22, 23, 41, 43). The present investigation describes the influence of the chemical environment on some physiological properties of C. botulinum type E.

Materials and Methods

Organisms. The following strains of C. botulinum type E were used: strains 996/62, 1217-764/64, 1537/62, 1653-1383/64, 2032/61, and 2835/61, from A. Johanssen, Public Health Authority Laboratory, Lund, Sweden; strains E6 and E104, from C. E. Dolman, University of British Columbia, Vancouver; and strains 3 pl, 3880 Hpl, 3889 pl/1/, and 3889 pl/2/, from G. Hobbs, Torry Research Station, Aberdeen, Scotland.

Culture media. The media used were proteose peptone medium (23), Robertsonss cooked meat medium (3), chemically defined medium D-Y (22), tributyrin agar, and egg yolk agar. The latter two were prepared by adding 2 ml of glycerol tributyrat and 10 ml of concentrated egg yolk emulsion (Oxoid), respectively, to the following base: 4 g of Proteose Peptone No. 3 (Difco), 2 g of yeast extract (Difco), 0.4 g of glucose, 60 mg of Na-thioglycolate, 2 ml of glycerol tributyrate, 0.2 g of NaCl, 2 g of agar, and distilled water to 100 ml, pH 7.4. Sterile stock solutions of glucose, Na-thioglycolate, tributyrat, and egg yolk emulsion were added aseptically to the sterile proteose peptone base. The chemically defined medium was prepared from stock solutions of the individual components and was sterilized by filtration. The complex media were sterilized by autoclaving for 15 min at 121 C. All stock solutions were stored in the dark at 4 C. Those not used within 2 weeks of preparation were discarded.

Toxin assay and neutralization tests. These were as described earlier (23).

Proteolytic activity. Proteolytic activity was estimated from the casein precipitating effect of proteolytic enzymes in casein agar as described by Sandvik (Thesis, Vet. College of Norway, Oslo, 1962). Preliminary experiments had shown this method to be the most sensitive one for estimating proteolytic activity. The casein agar was poured into petri dishes and the agar layer had a thickness of 2 mm. The culture filtrate to be tested was adjusted to pH 6.2 before 0.025 ml was deposited in wells (6-mm diameter). The petri dishes were incubated at 37 C for 20 hr.

Lipolytic and lecinthinase activity. Reactions in egg yolk agar as described by Willis (44, 45) and by McClung and Toabe (33) were used to demonstrate lipolytic and lecinthinase activity. Lipolytic activity was also indicated by clear zones in tributyrin agar.

Results

Efficiency of the synthetic medium. The chemically defined medium (D-Y) used for growth and toxin production of C. botulinum type E was
developed during the present investigation and its composition has been published elsewhere (22). The capacity of this medium to support growth and promote sporulation is further demonstrated in Fig. 1. It was also noticed that a minimal inoculum of about 100 cells/ml was needed to promote growth, and that the corresponding figure for proteose peptone medium was about 10 cells/ml.

The constancy of growth and toxin production after repeated subculturing in D-Y is shown in Fig. 2. Ten transfers did not affect growth or toxin production to any appreciable extent. The capacity of the synthetic medium D-Y to support growth in a different strain of C. botulinum type E was also tested. It was found that 8 strains of 12 tested supported growth.

Effect of choline and related substances on cell division. Choline has been shown to have an effect on cell division of C. botulinum type E (22). To understand better the mechanism of the effect of choline, a number of compounds structurally related to choline were tested regarding their capacity to substitute for choline in a synthetic medium. The results are shown in Table 1. Acetylcholine, N,N-dimethylethanolamine, and lecithin could substitute for choline, but not ethanolamine, N-methylethanolamine, or betaine. This indicated that N,N-dimethylethanolamine is the simplest compound necessary for normal cell division in the synthetic medium used.

Proteolytic activity. The potency of type E toxin can be greatly increased by a primary attack of proteolytic enzymes (15, 17, 18, 39). Continued exposure to these enzymes may bring about further fragmentation and partial inactivation of the toxin (14, 19, 40). This would explain the frequent decrease in toxicity of culture filtrate of C. botulinum type E on prolonged incubation, especially in the higher temperature range of growth (2, 4, 37). Since it is possible that proteolytic activity could be influenced by the presence of protein in the growth medium, it was decided to find out whether the proteolytic activity of a toxigenic strain of C. botulinum type E

![Graph](https://example.com/graph.png)

**FIG. 1.** Growth and sporulation of Clostridium botulinum type E (strain 1537/62) in D-Y medium. Incubation temperature, 30 C. Symbols: X, total number of cells/ml; O, number of sporulating cells/ml.

**FIG. 2.** Growth and toxin yield of Clostridium botulinum type E (strain 1537/62) in D-Y medium after subsequent transfers. First inoculum: 10° spores plus 3 ml of medium. Amount transferred: 0.1 ml of a full-grown culture to 3 ml of medium. Growth was estimated after 2 days at 30 C, toxin after 4 days. Symbols: X, growth; O, toxin, mean value of three mice. The standard deviation, calculated as $s = \sqrt{\frac{\sum(x - \bar{x})^2}{(n - 1)}}$, is shown by the vertical lines.

**TABLE 1.** Effect of choline and related compounds on cell division of Clostridium botulinum type E (strain 1537/62)

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Conc (mmole/ml)</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (D-Y with choline</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>omitted)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>0.36</td>
<td>+</td>
</tr>
<tr>
<td>Choline</td>
<td>0.36</td>
<td>+</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>0.36</td>
<td>+</td>
</tr>
<tr>
<td>N-methylethanolamine</td>
<td>0.36</td>
<td>+</td>
</tr>
<tr>
<td>N,N-dimethylethanolamine</td>
<td>0.36</td>
<td>+</td>
</tr>
<tr>
<td>Betaine</td>
<td>0.36</td>
<td>+</td>
</tr>
<tr>
<td>Lecithin</td>
<td>0.36</td>
<td>+</td>
</tr>
</tbody>
</table>
was different in the synthetic D-Y medium than in a complex medium, and whether such a difference would be reflected by a higher stability of the toxin formed in the synthetic medium.

Table 2 shows the relative proteolytic activity of culture filtrate of C. botulinum type E after incubation for various periods at 30 C. The media used were proteose peptone medium, cooked meat medium, and D-Y medium. The proteolytic activity could only be demonstrated in culture filtrates from the complex growth media but not from the synthetic D-Y medium (Table 2). Figure 3 shows the effect of incubation time on the toxicity of culture filtrate at different incubation temperatures. The media used were proteose peptone medium and D-Y medium. With incubation at 35 C or higher, a distinct decrease was found in the toxicity in the complex medium, but the toxicity in the D-Y medium was not affected to any appreciable extent. The toxicity of culture filtrates of C. botulinum type E grown in the D-Y medium was higher at 35 C than at 30 C. In proteose peptone medium the toxicity was higher at 30 C than at 35 C.

**Lipolytic and lecithinase activity.** With the methods used, lipase and lecithinase activity could be demonstrated in culture filtrates from the complex medium but not from the D-Y medium (Table 3). However, cells transferred from the D-Y medium and incubated on the complex test media recovered their capacity to produce lipid- and lecithin-destructive enzymes. Furthermore, when choline was replaced by lecithin in the D-Y medium, lecithinase activity could be demonstrated in the culture filtrate.

**Table 2.** Proteolytic activity of culture filtrate of Clostridium botulinum type E (strain 1337/62) grown in different media

<table>
<thead>
<tr>
<th>Age of culture (days)</th>
<th>Growth medium</th>
<th>D-Y</th>
<th>Robertsson cooked meat medium</th>
<th>Proteose peptone</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
<td>0*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0</td>
<td>0</td>
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</tr>
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<td>0</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>12</td>
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<tr>
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<td>0</td>
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<td></td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Results indicate diameter of precipitation zone in millimeters.

**Discussion**

The chemically defined medium D-Y used in this investigation supported growth and toxin formation by C. botulinum type E. On repeated subculture, the strain used did not show any degeneration phenomenon, such as restricted growth and toxin production or abnormal cell morphology. The capacity of the D-Y medium to induce sporeulation is noticeable, since the strain used did not sporulate freely in complex media.

In the synthetic D-Y medium, acetylcholine, N,N-dimethylethanolamine, and lecithin could replace choline without affecting normal cell division, but ethanolamine, N-methylethanolamine, or betaine could not. The fact that N,N-dimethylethanolamine, but not methionine and betaine containing labile methyl groups, could replace choline indicates that the role of choline is not that of transmethylation. The effect of acetylcholine could be understood if this substance is hydrolyzed by the organism to get choline. It is thought that choline acts as a precursor of certain choline-containing phospholipids in the cell membranes. Choline-containing phospholipids have so far been demonstrated in only relatively few species of bacteria (24, 30, 31, 46) and have not been found in the few species of Clostridium tested (20, 21). The formation of lecithin in the bottle media was only noted in the synthetic media from N,N-dimethylethanolamine, N,N-dimethylethanolamine, and choline is known to occur in lecithin-synthesizing bacteria (21, 29, 32). Our findings that ethanolamine and N-methyl-ethanolamine did not substitute for N,N-dimethylethanolamine or choline indicate that the biosynthesis of N,N-dimethylethanolamine from ethanolamine did not occur in C. botulinum type E grown in the D-Y medium. This is in agreement with Goldfine (20, 21), who found that N,N-dimethylethanolamine and choline could not be found by transmethylation in C. butyricum. However, we found that N,N-dimethylethanolamine could replace choline. This is most easily understood if one assumes that N,N-dimethylethanolamine is transmethylated to choline and consequently acts as a precursor of this substance. Another possibility is that N,N-dimethylethanolamine may be a constituent of certain phosphatidies in the cell membranes and gives rise to normal cell division. This substance has been found in phosphatides from Agrobacterium tumefaciens (32) and from a choline-requiring mutant of Neurospora crassa unable to synthesize choline (25), but not in C. butyricum (20).

The action of choline on the cell division of C. botulinum type E may thus be due to its role as a
constituent of certain choline-containing phospholipids in the cell membranes, which are known to be involved in the cell division of bacteria. However, if this assumption is true, further investigation may show that choline really is a constituent of the cell membranes of C. botulinum type E grown in the D-Y medium. It is also possible that the function of choline and N,N-di-methyllethanolamine in the cell division of C. botulinum type E is entirely different from their known roles in synthesis of phosphatides in bacteria.

Toxigenic strains of C. botulinum type E are generally believed to be nonproteolytic. It has, however, been claimed that certain strains might have a weak proteolytic activity (A. Skulberg, Thesis, Vet. College of Norway, Oslo, 1964). In our experiments, strain 1537/62 showed proteolytic activity after 8 days in a complex medium but not in the synthetic medium. It seems that the decrease in toxicity on prolonged incubation at 35 and 37 C in the complex media is due to the proteolytic inactivation of toxin already formed.

In our experiments, neither lipase nor lecithinase activity could be demonstrated in culture filtrate from the D-Y medium. On the other hand, when cells from the D-Y medium were incubated on the egg yolk agar and tributyrin agar, they recovered their capacity to form lipolytic and lecithinase activity. When choline was replaced by lecithin in the D-Y medium, lecithinase but not lipase activity could be demonstrated. Our results indicate that the lipid as well as the lecithin-destructive enzymes are adaptive. This seems to support the view expressed by Cutchins et al. (11) that bacterial lipases are adaptive in nature.
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