and inositol was no greater than in the control medium which contained no added carbohydrate.

Growth and toxin synthesis by strain JDT-IV, which may be taken as representative for all the strains tested, are presented graphically in figure 1 and 2, respectively. Although equal growth rates were observed during the exponential growth phase with all the carbohydrates, maximum growth was achieved only with glucose and maltose. The degree to which growth was repressed with the other carbon compounds when compared to glucose, however, was not sufficient to account for the extreme differences in toxin production. It can also be noted that autolysis of the culture did not go to completion unless glucose or maltose was included in the growth medium. Sonic disintegration of the intact cells, which remained after 72 hr of incubation in the media containing carbohydrates other than glucose and maltose, did not result in the increased toxicity of the supernatant fluid showing that the toxin was not present intracellularly.

### Physiology of Toxin Production by *Clostridium botulinum* Types A and B

#### III. Effect of pH and Temperature During Incubation on Growth, Autolysis, and Toxin Production

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The ever present hazard of botulinus intoxication has stimulated continuing research by various segments of the food industries. The fact that commercially prepared foods have been only rarely incriminated in botulinus outbreaks during the past three decades is testimony to the effectiveness of the research which has been carried out to determine the proper measures for avoiding such occurrences. The literature is voluminous regarding the upper and lower limits of pH and temperature which will support the growth of *Clostridium botulinum* in various natural food substrates and artificial media. However, these investigations are for the most part concerned with an all or none phenomenon, the organism multiplies and produces toxin or it does not. Ohye and Scott (1953, 1957) did quantitative growth determinations of *C. botulinum* types A, B, and E at various temperatures but did not attempt to correlate this with toxin production. Since autolysis has been shown to be correlated with the liberation of toxin by *C. botulinum* (Boroff, 1955; Kindler et al., 1955; Bonventre and Kempe, 1959), it was considered that a quantitative study concerning the effects of pH and temperature incubation on the growth, autolysis, and toxin production by *C. botulinum* would be valuable from both a practical and theoretical point of view.

#### Materials and Methods

*C. botulinum* type A strain JTD-IV\(^2\) was used in this investigation. Growth was measured turbidimetrically, and toxin assayed as described previously by Bonventre and Kempe (1959).

**pH of medium.** The complete medium described by Bonventre and Kempe (1959) for the cultivation of all type A strains of *C. botulinum* was freshly prepared and

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PHYSIOLOGY OF TOXIN OF C. BOTULINUM. III

adjusted to pH values between 4.5 and 8.3. Preheating of the medium was carried out under flowing steam and cooled before adjusting to the desired pH. This was found to be necessary since the pH often changed upon sterilization if preheating was not carried out. The media were dispensed into matched 15/150 tubes in 10-ml quantities so that turbidimetric measurements of growth could easily be made. One-tenth ml of an 18-hr culture of the organism was inoculated into a sufficient number of tubes so that a culture at each level of hydrogen-ion concentration could be taken after suitable incubation periods and assayed for toxin. This procedure was found to be more advantageous than working with a single culture at each pH level since manipulation during sampling at times resulted in a cessation of growth due to over oxygenation.

Temperature of incubation. The procedure of inoculating the organism into matched test tubes containing 10 ml quantities of the complete medium was also adopted for these experiments in which the temperature of incubation was the experimental variable. The medium was adjusted to a pH of 6.8 and growth and toxin synthesis of cultures incubated between 4 and 55 C were recorded. The precision of temperature control in the incubators was ±1 C. Experiments conducted below room temperature were carried out by incubation in cold running water which fluctuated between 10 and 18 C.

RESULTS AND DISCUSSION

Growth and toxin synthesis by C. botulinum strain JTD-IV are shown in figures 1 and 2, respectively. Optimal growth of the organism was obtained between pH 5.5 and 7.0 and submaximal growth between 7.0 and 8.3. At pH values of 5.3 and below, the organism was not able to initiate growth. The value is higher than the minimum pH at which certain strains of C. botulinum have been reported to be capable of growth and toxin production. Gunnison and Meyer (1929) described an outbreak of botulism from home canned pears, the syrup of which had a pH of 3.86. However, since a lactobacillus was also isolated, the authors point out that the acids produced by the lactobacilli may have increased the acidity of the syrup after the botulinum toxin was produced. Dozier (1924) and Townsend et al. (1954) reported growth and toxin production by some strains of C. botulinum at pH levels slightly under 5.0, but found that the absolute minimum varied considerably from strain to strain and medium to medium.

The autolytic process was most rapid between pH 6.5 and 7.0, indicating that the autolytic enzymes have a rather narrow pH range for optimum activity. Maximum toxin titers were obtained between pH 5.5 and 8.0, but it was found that the toxin was much more stable at pH 6.5 and below. Although an alkaline environment resulted in an inactivation of the toxin, it did not seem to affect its synthesis. It is interesting to note that the toxin was most stable at pH 5.5 where the rate of autolysis was comparatively slow. This suggests that the stability of the toxin may possibly be increased by virtue of large quantities being bound intracellularly for longer periods of the time than in an environment where autolysis proceeds very rapidly.

The pH range in which C. botulinum strain JTD-IV was able to initiate growth and synthesize toxin was found to lie between pH 5.5 and 8.3. The rate at which toxin was synthesized did not vary markedly with pH. However, the rate limiting factors of the quantity of
toxin found extracellularly appeared to be the degree to which the culture had autolyzed and to the stability of the toxin in its environment. Since the autolytic enzymes appear to be most active between pH 6.5 and 7.0, cultures grown at these hydrogen ion concentrations liberated the greater part of their toxin shortly after maximum growth was achieved. It is well known that this protein toxin is unstable in an alkaline environment and thus, above pH 6.8, a considerable quantity was inactivated. It can be seen that, if the toxicity of the extracellular culture fluids were determined at only one point in the growth cycle, after 48 hr for example, one might be led to the erroneous conclusion that more toxin is produced between pH 6.5 and 6.8

Figure 3. Effect of incubation temperature on growth of Clostridium botulinum strain JTD-IV.

Figure 4. Effect of incubation temperature on toxin synthesis by Clostridium botulinum strain JTD-IV.
than at any other level of hydrogen ion concentration. Interpretation of data of this nature may in fact be one of the contributing factors for the conflicting reports found in the literature concerning the kinetics of botulinum toxin production.

The rates of growth, autolysis, and toxin synthesis varied considerably with the temperature of incubation (figures 3 and 4). Between 28 and 40°C, maximum growth, autolysis, and toxin synthesis were obtained although, as would be expected, the rates at which the processes occurred increased with increasing temperature. In all cases, maximum toxicity of the culture filtrates was reached only after autolysis was complete. Temperatures between 20 and 26°C supported growth adequately but not optimally. At these temperatures lysis was retarded and, unlike cultures which had completely autolyzed, prolonged incubation resulted in a gradual increase in the quantity of toxin found extracellularly. The maximum temperature which supported growth was 48°C. At this temperature, however, although some growth could be measured turbidimetrically, no increase in the toxicity of the filtrates could be detected. Furthermore, the toxicity conferred on the cultures by the initial inoculum disappeared during incubation at 48°C. Since the organism was able to multiply at this elevated temperature, it is not likely that toxin synthesis was completely inhibited. The evidence suggests that any toxin which was elaborated was inactivated by gradual denaturation.

No growth or toxin synthesis was demonstrable at temperatures between 4 and 10°C (refrigeration temperatures). However, when cultures were incubated in the water bath which fluctuated between 10 and 18°C (food handling temperatures), growth and toxin synthesis were noted. At these low temperatures the toxin is quite stable and, consequently, prolonged incubation resulted in a gradual increase in toxicity. After 190 hr of incubation, the culture filtrates contained 5000 MLD per ml. The tremendous expansion of the frozen food industries and the possibility that irradiation pasteurization of some foods may be adopted makes this observation a significant one since this is the approximate temperature range at which foods may be kept and manipulated in retail establishments. However, the results suggest that the hazard of botulinus intoxication resulting from nonsterilized foods is likely only if the storage temperatures are not adequately controlled.

**Summary**

The pH of the culture medium did not affect the synthesis of botulinum toxin within the limits which supported the growth of *Clostridium botulinum* strain JTD-IV (pH 5.3 to 8.3). The hydrogen ion concentration indirectly affected the toxicity of culture filtrates by virtue of the narrow pH optimum for the autolytic enzymes and the instability of the toxin above pH 6.8. The maximum temperature at which growth of the organism occurred was 48°C; at this temperature the toxin was gradually inactivated. At 10°C or below, metabolic activity was not detectable. Temperatures fluctuating between 10 and 18°C partially supported growth and toxin synthesis. The implications of these observations are discussed.

**REFERENCES**


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