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EFFECT OF CARBOHYDRATE ON TOXIN PRODUCTION BY *CLOSTRIDIUM BOTULINUM* TYPE A

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INTRODUCTORY

During the course of studies on toxin production by *Clostridium botulinum* type A, it was noted that addition of a certain carbohydrate in a growth medium enhanced considerably the toxicity of culture fluid of the organisms[3,6,9]. The present study was undertaken to define the correlation of carbohydrate to growth and to toxin production by the organisms.

MATERIALS AND METHODS

The strain used was *Cl. botulinum* type A No. 38. A medium consisting of 1.0 per cent proteose peptone (Difco) and 1.0 per cent yeast extract (Difco) was employed for the cultivation of the organisms. After sterilization, the carbohydrate and sodium thioglycollate were added to make final concentrations of 1.0 and 0.05 per cent. Initial pH of the medium was adjusted to 7.2. Detailed procedures of each experiment will be described later.

Growth of the organisms was measured turbidimetrically with an electrophotometer at 530 mμ. Toxicity was established by intraperitoneal injection of the diluted samples into white mice of about 20 g, and titre of toxin was expressed in terms of MLD.

EXPERIMENTAL AND RESULTS

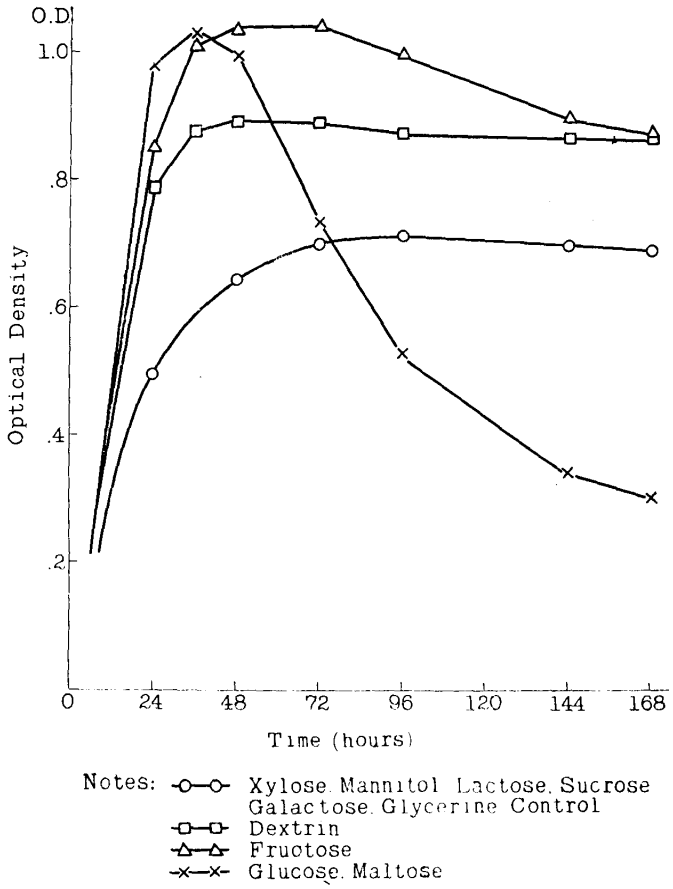
1. Growth and Toxin Production in the Presence of Various Carbohydrates

Growth and toxin production by *Cl. botulinum* type A No. 38 were tested in media supplemented with various carbohydrates. Test tubes, in which the medium supplemented with test carbohydrate was placed, were de-oxygenated by immersion in a boiling water bath for 10 minutes, cooled, inoculated with 18-hour culture of the organisms, and incubated at 30°C for 7 days. Growth was measured periodically and toxin was assayed on 7-day culture filtrate.

Results are shown in table 1 and figure 1. It was found that glucose, maltose and fructose supported toxin production fully. Of the other carbohydrates tested, only dextrin partially supported toxin production. Titres of toxin in the presence of other carbohydrates were no greater than in the control medium; they were below 1,000 MLD.

Considerable growth of the organisms was observed in the absence of utilizable carbohydrate, although maximum growth was achieved only with glucose, maltose and fructose. The degree to which growth was repressed with no carbohydrate when compared to growth

FIGURE 1. *Growth of Cl. botulinum Type A No. 38 in the Presence of Various Carbohydrates*



with glucose, however, was not sufficient to account for the extreme differences in toxin production.

Final pH of cultures had a tendency to be more acidic than initial pH of 7.2 in the presence of utilizable carbohydrates and to be more alkaline in the absence of them.

Growth curves of the organisms with glueose and maltose were characterized by rapid descent following after a maximum growth phase. This seems to suggest that obvious autolysis of the culture does not take place unless glucose or maltose is included in the culture medium.

From the pattern obtained through these results, the following three suggestions could be expected regarding the reason for extreme differences in toxicity: First, there have been some indications that the alkaline environment resulted in the inactivation of toxin. It is not unreasonable to assume that a considerable quantity of toxin could be produced even in the absence of glucose and it would be immediately denaturated in alkaline environment. Second, this suggestion is based on an autolysis. BOROFF, STONE, and BONVENTRE and KEMPE[1] reported that autolysis has been established as an important mechanism for the liberation of exotoxin from the cells. The fact, visible autolysis was not observed in the absence of glucose or maltose, may suggest an incompleteness of toxin liberation from the cells in these cultures.

TABLE 1. *Toxin Production by Cl. botulinum Type A No. 38 in the Presence of Various Carbohydrates*

CARBOHYDRATE	FINAL pH	TOXIN TITRE (MLD/ml)
Dextrin	6.5	40,000
Mannitol	7.8	< 1,000
Lactose	7.8	< 1,000
Sucrose	7.8	< 1,000
Maltose	5.6	160,000
Glucose	5.4	160,000
Fructose	5.0	160,000
Galactose	7.8	< 1,000
Xylose	7.8	< 1,000
Glycerine	7.8	< 1,000
Control (No carbohydrate)	7.8	< 1,000

Third, it was suggested by CLIFTON that *Cl. botulinum* could obtain its energy by a series of reactions following the Stickland reaction. If synthesis of toxin takes place only when carbohydrate is utilized by the organisms as an energy source, less toxin in the absence of any carbohydrate would be a matter of course.

Following studies were undertaken on the basis of these points.

2. Effect of Initial pH on Growth and Toxin Production by the Organisms

Two kinds of media, supplemented with glucose and without glucose, were placed in test tubes, adjusted to pH values between 5 and 8, and inoculated. Growth was measured periodically. After 7-day incubation, titre of toxin and final pH in each tube were examined.

Results are shown in table 2 and figure 2. In the cultures with glucose, little difference in quantity of the organisms was observed within the test pH range, although the time necessary to reach the maximum growth was slightly different between them. In the cultures with no glucose, maximum growth was obtained at pH 7 and 6, while growth at pH 8 and 5 was somewhat less than that at pH 7 and 6. On the other hand, titres of toxin in comparatively alkaline cultures with glucose and no glucose were considerably less than those of acidic ones. It remains to be ascertained, however, that the inferiority of toxin titre in alkaline environment resulted from either synthesis of less toxin under such a condition or inactivation of toxin had been once synthesized.

3. Effect of pH on Appearance of the Toxin into an Extracellular Environment from the Cells

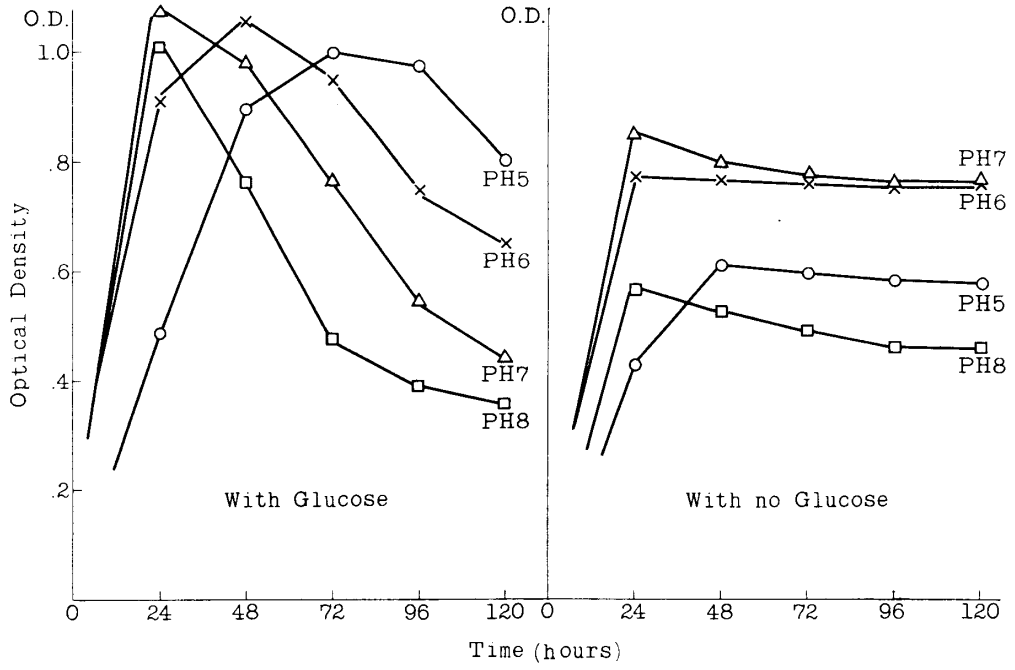
KINDLER, MAGER and GROSSOWICZ[6] and BONVENTRE and KEMPE[2] stated in their reports that *Cl. botulinum* synthesized a great part of toxin intracellularly prior to achievement of the maximum growth and liberated it into the extracellular environment through a certain process, which seemed to be an autolysis.

The organisms were harvested separately from cultures with glucose and without glucose

TABLE 2. *Effect of pH on Toxin Production*

INITIAL pH	WITH GLUCOSE		WITH NO GLUCOSE	
	Final pH	Toxin Titre (MLD/ml)	Final pH	Toxin Titre (MLD/ml)
5.0	5.0	80,000	7.0	4,000
6.0	5.2	80,000	7.6	1,000
7.0	5.4	160,000	8.0	< 500
8.0	6.0	20,000	8.6	< 500

FIGURE 2. *Growth of Cl. botuinum Type A No. 38 at Various pH Levels*



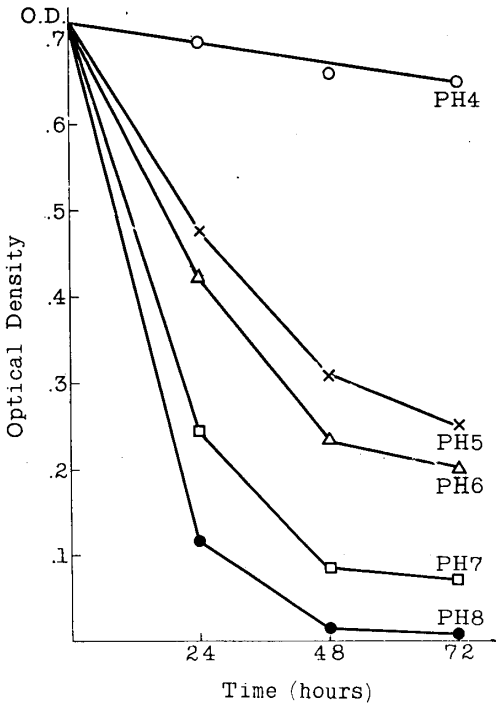
at the end of the logarithmic growth phase; at that time little toxin was found in culture filtrates. The organisms were washed thoroughly with physiological saline containing sodium thioglycollate and resuspended in a small volume of fresh medium. A fixed volume of two kinds of cell suspension was added to each tube, in which fresh medium with pH between 4 and 8 and penicillin in final concentration of 1,000 unit/ml had been placed previously. Then, the tubes were incubated at 30°C. The degree of lysis and toxicity of medium were measured periodically.

Table 3 shows the toxicities of supernatant fluids of each cell suspension after 24-hour incubation. Maximum titre of toxin was found at pH 5 and 6 in both suspensions using the cells originated from glucose and glucose-less cultures. Titres of toxin at other pH values were less than those at pH 5 and 6; that which was especially remarkable in alkaline pH. Similar findings were also obtained when 1/20 M phosphate buffer at various pH values was

TABLE 3. *Effect of pH on Toxin Liberation from the Cells in the Presence of Penicillin*

pH	TOXIN TITRE (MLD/ml)	
	With Glucose	With no Glucose
4.0	4,000	2,000
5.0	32,000	4,000
6.0	32,000	4,000
7.0	< 4,000	< 500
8.0	< 2,000	< 500

FIGURE 3. *Lysis of the Cells at Various pH Levels*



used as the dispersion medium. During the incubation period, the organisms could not grow because of the existence of penicillin nor could they synthesize the toxin intracellularly.

Figure 3 shows the degree of lysis at various pH values of the organisms from the culture with glucose. As seen in the figure, at pH 4 little autolysis occurred even after 72 hours incubation. This presented the question whether a certain quantity of toxin synthesized intracellularly could not be liberated at this pH level but remains in the cells. In order to solve the question, contents of every tube were adjusted to pH 5.8 again and reincubated at 30°C. After 24 hours incubation, the toxicity of each content was assayed. It was found that the toxicity of the medium at pH 4 which had been 4,000 MLD previously was increased to 32,000 and toxicity of media at pH 7 and 8 was still the same as each previous value.

From the results obtained in this run of experiments, it would be suggested that the cause of low toxin titre in alkaline culture was not the synthesis of less toxin but the inactivation of toxin.

The suggestion was also supported by the following experiment. The organisms were cultivated in media with glucose at pH 6, 7 and 8. These pH values were kept constant by occasional addition of diluted NaOH solution through the cultivation period. The organisms in each culture were harvested at the end of logarithmic growth phase, suspended separately in 1/20M phosphate buffer pH 5.8 and incubated at 30°C for 24 hours. No difference in toxicity between the suspensions was observed.

4. Toxicities of Cultures Supplemented with Various Energy Sources at Fixed pH Value

A considerable quantity of toxin was demonstrable as shown in table 3, even in the absence of glucose, provided the pH of the growth medium was carefully maintained at a favorable level. This seemed to suggest the possibility that glucose which supplied as an energy source may be replaced with certain amino acids or their derivatives and that a corresponding amount of toxin to that of with glucose may be obtained in these latter media.

With the view of testing the possibility, the organisms were cultivated in the medium supplemented with casamino acids or various types of peptone. Through the incubation period, pH of culture was kept at about 6.0 by occasional addition of diluted NaOH solution.

TABLE 4. *Replacement of External Glucose with Amino Acids Containing Materials*

MATERIAL (Final 1%)	RELATIVE GROWTH (%)	TOXIN TITRE (MLD/ml)
Control	73	20,000
Glucose	100	160,000
Casamino Acids	90	40,000
Proteose Peptone	98	80,000
Mikuni Peptone	95	40,000
Mikuni Peptone (For Toxin Production)	98	80,000
Yenjo Peptone	88	40,000

Results are shown in table 4. From the table, it is evident that toxicities of media supplemented with casamino acid and peptone are considerably higher than toxicity of the control, although a little difference exists in toxicity between the glucose culture and the culture with substituted energy source. Consequently, it may be suggested that glucose is not always required essentially for toxin production by the organisms.

DISCUSSION

There have been some reports that although considerable growth of *Cl. botulinum* type A was possible even in media with no glucose, generally the titre

of toxin in such culture was less than that in a culture with glucose. The present study also supported these findings. The differences in toxicity between the cultures with glucose and without glucose, at least in part, would result from the differences in pH level occurring during cultivation. That is, even in the absence of glucose considerable amounts of toxin are synthesized intracellularly by the organisms and liberated into the environment medium, but they are immediately inactivated in alkaline environment. Toxin synthesis within the cell did not seem to be affected by alkaline environment. KINDLER et al.[7] and BONVENTRE and KEMPE[3] stated that glucose is essential for optimum toxin synthesis by the organisms. The data obtained in the present experiments, however, indicated that a certain carbohydrate is the most favourable energy source for toxin production, but it is not essential, because glucose, the most effective carbohydrate, can be replaced with other substances such as casamino acids and peptones to some extent. Consequently, it could be considered that the principal factor governing toxin production is not the quality of energy source but the quantity of utilizable energy and that the toxin is produced by the organisms only under the condition of availability of rich energy.

No great autolysis of the culture was found unless a certain carbohydrate was included in the growth medium. STONE, BOROFF and BONVENTRE and KEMPE[1] reported that autolysis is the main process of toxin liberation by the organisms. In the present experiments, however, it was found that in the culture with no glucose in which visible autolysis could not be observed, the toxin could be easily liberated into the extracellular environment. Consequently, whether autolysis is an essential mechanism of toxin liberation or not is still in question, although it is ascertained that the great part of toxin synthesized intracellularly is liberated during the catabolic phase of the organisms.

Since the present experiments have been attempted only with one strain of *Cl. botulinum* type A, it is quite uncertain whether suggestions described above could be applied to other strains or not.

SUMMARY

Growth of *Cl. botulinum* type A and production of toxin by it were promoted fully by external glucose, maltose and fructose in tested carbohydrates. Considerable growth of the organisms was obtained even in the absence of these carbohydrates but only an extremely low level of toxicity was demonstrable in the culture. The cause for such result was investigated, and as a result, it was suggested that the less toxicity in the absence of glucose was partly a result from the inactivation of synthesized toxin, occurring in alkaline environment, and partly from the insufficiency of any suitable energy source required for synthesis of more toxin.

Additionally, some conditions governing the production of toxin in the culture were discussed.

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