TOXIN PRODUCTION BY CLOSTRIDIUM PERFRINGENS

I. SYNTHESIS OF α- AND β-HEMOLYSINS, LECITHINASE AND PROTEINASE IN A SYNTHETIC MEDIUM

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Received for publication April 1, 1957

Continued interest in the disease enterotoxemia of sheep and of cattle, which is believed to be related to excessive multiplication and toxin production of intestinal Clostridium perfringens, has stimulated this investigation of the nutritional factors necessary for toxin production by various types of this organism. The ultimate goal of this work is the production of an immunizing agent having greater efficacy than those now available. A more detailed knowledge of the specific nutritional factors required for toxin production appears essential if it is hoped to produce for isolation the immunogenic factors of the many toxins known to be synthesized. Economic considerations make it unlikely that use of a reproducible, synthetic and protein free medium would be feasible in the large scale production of toxins. However, its use seems extremely valuable as a tool for the acquisition of a better understanding of growth and toxigenic factors and for the greater simplicity of isolating toxins (enzymes) from a protein free medium. The importance of sharp differentiation between growth and toxigenic properties has been emphasized by Van Heyningen (personal communication).

The chemically defined medium reported by Boyd et al. (1948) supports luxurious and rapid growth of several strains of C. perfringens incubated at 43 to 46 C. Under these conditions the production of exotoxins was not detected by in vitro assays. Recently, Duff and Costerton (1956) have shown that the above medium can support the production of lecithinase by C. perfringens.

This report presents experimental evidence for growth and toxin production in the synthetic medium, previously shown to support no toxinogenesis, by 6 strains of C. perfringens, including strain BP6K used by Boyd et al. (1948).

MATERIALS AND METHODS

The composition of the medium employed in this study has been mentioned (Boyd et al., 1948). All organisms, including two of type A, one each of types B and C and two of type D cultures of C. perfringens were carried serially in the defined medium. Transfers were made at least once every 2 weeks. One liter of the medium was prepared at a time. This was dispensed in 10 ml quantities in the small screw cap tubes (125 by 17 mm O. D.) used to carry the stock cultures. Toxin production studies were performed in larger screw cap tubes (150 by 27 mm O. D.) which contained 40 ml of the medium. With the exception of one experiment, glucose was sterilized by filtration and added aseptically to the sterile medium to give a final concentration of one per cent.

For all experiments an inoculum of 5 per cent by volume of a 16 to 20 hr vigorously growing culture was used. After an incubation period of 7 hr at either 37 or 43 C, two 10 ml aliquots were taken for duplicate washed cell nitrogen determinations, 3 to 4 ml for pH measurement with a glass electrode and the remainder was centrifuged to pack the cells so that an optically clear supernatant could be used for the assay of toxins.

Preparation of the red blood cell suspension. Sheep blood was collected in Alsever's solution, washed twice with saline and resuspended in buffer to give a final concentration of 2 per cent by volume. Suspensions were prepared fresh on the day of use.

Determination of α-hemolysin. One ml aliquots of the supernatant were diluted serially in a series of tubes containing 1 ml of M/100 acetate buffer of pH 6.0 (Burrows, 1951). To each tube 1.5 ml additional buffer and 0.5 ml of the red...
cell suspension were added. The cells were mixed by inversion of the tubes and following incubation at 37 °C for 30 min the tubes were refrigerated overnight since it has been established previously that α-hemolysin is a hot-cold lytic agent (Macfarlane, 1950). The tubes were centrifuged so as to pack the cells and the clear supernatant was carefully decanted and used to determine hemoglobin concentration in a Klett-Summerson colorimeter using a 540 μm filter. The 50 per cent endpoint was determined from standards prepared for each suspension of red cells. To differentiate between growth and toxigenic factors, results are expressed in terms of hemolytic units (HU) per ml of culture medium and HU per mg of washed cell nitrogen. The HU in this paper is defined as that quantity of hemolysin causing the release of 50 per cent of the hemoglobin contained in 0.5 ml of a 2 per cent suspension of sheep red blood cells.

Determination of θ-hemolysin. θ-Hemolysin was determined by a modification of the method used by Roth and Pillemer (1955). One ml aliquots of cell free supernatant were diluted, as was done for the α-hemolysin determinations, in sodium phosphate-sodium chloride buffer of pH 6.8. One-half ml of 0.1 M L-cysteine hydrochloride, adjusted to pH 6.8 in the buffer was added and the tubes were incubated for 15 min in a 37 °C water bath. One-half ml of a 2 per cent suspension of red cells was added and the cells were mixed by inversion before reincubation for 30 min at 37 °C. For convenience, these tubes were refrigerated overnight along with the α-hemolysin tubes before the 50 per cent hemolytic endpoint was determined. Results are reported in a manner analogous to that used for α-hemolysin.

Preparation of the fifty per cent hemolytic standards. The 50 per cent hemolytic standards for the hemolysins were prepared by adding 0.5 ml of the cell suspension to 2.5 ml of water for the α-hemolysin standard and to 2.0 ml of water plus 0.5 ml buffered cysteine solution in buffer for the θ-hemolysin standard. After refrigeration with the assay tubes, 3.0 ml additional water was added to give a hemoglobin color equivalent to that for 50 per cent hemolysis.

Lecithinase determinations. A solution of lecithovitellin (LV) was prepared according to the method given by Wagner (1949). The yolk of one fresh chicken egg was mixed with 200 ml of physiological saline and heated at 56 °C for 30 min. This solution was filtered through a series of Seitz filters until a light yellow, faintly opalescent solution was obtained. The test itself was performed by adding 1 ml of the cell free culture supernatant to 1 ml LV solution and incubating the resulting solution for 1 hr at 37 °C. The tubes were refrigerated overnight and turbidity was measured at 420 μm in the colorimeter after 4 ml saline was added and the tubes mixed. The qualitative results are expressed as follows:

Scale reading for turbidity in assay tube

Scale reading for turbidity in control tube

Determination of proteinase. The determination of this enzyme or possibly several enzymes was accomplished by using a modification of the method described by Bidwell and Van Heyningen (1948). Seventy mg of 120 mesh azocoll (Oakley et al., 1948) was added to 15 ml tubes. One ml of the culture supernatant and 5 ml of buffer at pH 7.4 (equal volumes of m/15 phosphate buffer and 1 per cent sodium chloride) were added. The tubes were tightly stoppered, mixed and completely submerged for 1 hr at a 37 °C water bath. The tubes were mixed several times during the period of incubation. After 1 hr, the solution was filtered to stop enzyme action and color development was measured at 520 μm in the colorimeter. Results are expressed as follows:

Scale reading for color in assay tube

Scale reading for color in control tube

Use of azocoll is a valid method for the demonstration of various proteinases although it is not specific for collagenase detection since Neuman and Tytell (1950) have shown that other proteolytic enzymes may also attack azocoll. No effort was made here to differentiate between λ- and θ-toxins, both of which are proteolytic in their action.

Washed cell nitrogen determinations. Duplicate 10 ml aliquots from the well-mixed 7 hr culture were centrifuged at a speed sufficient to pack the cells. The clear supernatant was carefully removed through a pasteur pipette connected to a water vacuum pump. The packed cells were twice resuspended in distilled water and sedimented. The cells, resuspended in 3 to 4 ml of water, were transferred quantitatively into 100 ml Kjeldahl digestion flasks. The cells were digested in the presence of a selenized granule, sulfuric acid, cupric sulfate and potassium sulfate. After digestion, the ammonium-nitrogen was
ence to due indicates that This and differed pH values from distilled steam (Scientific Supplies, England). With the exception of an excess of sodium hydroxide. The ammonia was collected in saturated boric acid and titrated with N/70 sulfuric acid in the presence of a methyl-red bromocresyl-green indicator.

RESULTS AND DISCUSSION

Hemolysin production. Toxin yields in Boyd's original medium are contrasted with the decreased yields obtained when the glucose was autoclaved in the medium at a pH of 7.2 (table 1). The caramelization which occurred definitely resulted in reduced toxin production. This effect is in complete agreement with the inhibition of growth observed under similar circumstances by Boyd et al. (1948). Growth inhibitors are formed concomitant with the process of caramelization. With the exception of culture no. 54, final growth pH values between the two media were above 6.0 and differed by no more than 0.6 of a pH unit. This indicates that differences in toxin yields are due to inhibition of growth and not to pH effects. Washed cell nitrogen determinations of strain BP6K were 0.64 mg/10 ml in the original medium contrasted to 0.21/10 ml in the caramelized glucose medium.

The effect of the incubation temperature on toxin yields is shown in table 2. In each case the hemolysin produced per ml of culture is as great or greater at 43 than at 37 C. This difference, however, is due to the growth stimulating effect of the higher temperature rather than a toxigenic effect since the toxin yields, calculated per mg of cell nitrogen, are decidedly greater at 37 C. The final pH values after incubation at 43 C were below 6.0 and it is possible that such low values may either inactivate toxin formed or inhibit its formation in this defined medium, although Gale and Van Heyningen (1942) reported that α-toxin is produced in the pH range 5.5 to 7.0 in a salt-beef extract medium.

An attempt to distinguish between pH inhibition and toxin inactivation was made by growing 3 cultures at 37 and 43 C in the presence and absence of added glucose (table 3). In the absence of added glucose, culture BP6K produced detectable θ-hemolysin and a negligible amount of α-hemolysin when grown at 37 C whereas culture 54 produced θ-hemolysin at both 37 and 43 C.

TABLE 1

Comparison of toxin yields at 37 C when glucose is autoclaved with the medium and when it is sterilized by filtration and added aseptically to the autoclaved medium

<table>
<thead>
<tr>
<th>Culture</th>
<th>Type</th>
<th>α-Hemolysin</th>
<th>θ-Hemolysin</th>
<th>Lecithinase</th>
<th>Proteinase</th>
<th>Final Growth pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HU*/ml</td>
<td>HU/mg N</td>
<td>HU/ml</td>
<td>HU/mg N</td>
<td></td>
</tr>
<tr>
<td>BP6K</td>
<td>A</td>
<td>357</td>
<td>5,580</td>
<td>385</td>
<td>6,000</td>
<td>0</td>
</tr>
<tr>
<td>1156</td>
<td>A†</td>
<td>66</td>
<td>700</td>
<td>63</td>
<td>650</td>
<td>0</td>
</tr>
<tr>
<td>CN372</td>
<td>B</td>
<td>53</td>
<td>1,000</td>
<td>48</td>
<td>910</td>
<td>0</td>
</tr>
<tr>
<td>215</td>
<td>C†</td>
<td>†</td>
<td>†</td>
<td>†</td>
<td>220</td>
<td>0</td>
</tr>
<tr>
<td>324</td>
<td>D†</td>
<td>110</td>
<td>1,800</td>
<td>167</td>
<td>2,780</td>
<td>0</td>
</tr>
<tr>
<td>54</td>
<td>D†</td>
<td>530</td>
<td>2,700</td>
<td>333</td>
<td>1,680</td>
<td>0</td>
</tr>
</tbody>
</table>

* HU = hemolytic units.
† Provisional classifications based on toxin neutralization tests with specific typing sera obtained from the Wellcome Laboratories, England.
¶ Less than 50 per cent hemolysis in the first dilution tube was considered negligible.
Comparison of toxin yields at incubation temperatures of 37 and 43°C

<table>
<thead>
<tr>
<th>Culture</th>
<th>Type</th>
<th>Temperature</th>
<th>α-Hemolysin</th>
<th>β-Hemolysin</th>
<th>Lecithinase</th>
<th>Proteinase</th>
<th>Final pH</th>
</tr>
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<tbody>
<tr>
<td>CN372</td>
<td>B</td>
<td>37</td>
<td>18</td>
<td>720</td>
<td>11</td>
<td>440</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>30</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>5.9</td>
</tr>
<tr>
<td>215</td>
<td>C</td>
<td>37</td>
<td>6</td>
<td>158</td>
<td>8</td>
<td>218</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>27</td>
<td>29</td>
<td>0.8</td>
<td>0.3</td>
<td>5.9</td>
</tr>
<tr>
<td>324</td>
<td>D</td>
<td>37</td>
<td>173</td>
<td>3,260</td>
<td>100</td>
<td>1,800</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>173</td>
<td>1,440</td>
<td>131</td>
<td>1,000</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* HU = hemolytic units.

Table 3
Comparison of toxin yields when cultures were incubated at 37 and 43°C in the presence or absence of added carbohydrate

<table>
<thead>
<tr>
<th>Culture</th>
<th>Type</th>
<th>Temperature</th>
<th>Glucose</th>
<th>α-Hemolysin</th>
<th>β-Hemolysin</th>
<th>LV*</th>
<th>Proteinase</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP6K</td>
<td>A</td>
<td>37</td>
<td>+</td>
<td>666</td>
<td>6,400</td>
<td>3.2</td>
<td>1.3</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37</td>
<td>-</td>
<td>§</td>
<td>§</td>
<td></td>
<td>0</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>+</td>
<td>1,050</td>
<td>5,900</td>
<td>0.1</td>
<td>0.1</td>
<td>7.1</td>
</tr>
<tr>
<td>1156</td>
<td>A</td>
<td>37</td>
<td>+</td>
<td>12</td>
<td>400</td>
<td>0.4</td>
<td>0</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>-</td>
<td>§</td>
<td>§</td>
<td></td>
<td>0</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>+</td>
<td>23</td>
<td>365</td>
<td></td>
<td>0.2</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>-</td>
<td>§</td>
<td>§</td>
<td></td>
<td>0</td>
<td>7.0</td>
</tr>
<tr>
<td>54</td>
<td>D</td>
<td>37</td>
<td>+</td>
<td>176</td>
<td>1,140</td>
<td>1.2</td>
<td>0.2</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>+</td>
<td>43</td>
<td>250</td>
<td>0.1</td>
<td>0.2</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>-</td>
<td>§</td>
<td>§</td>
<td></td>
<td>0</td>
<td>7.2</td>
</tr>
</tbody>
</table>

* LV = lecitho-vitellin.
† HU = hemolytic units.
‡ Not determined for 43°C incubation in absence of added sugar.
§ Less than 50% hemolysis in the first dilution tube was considered negligible.

The final pH in these two instances was above 7.0. Gale and Van Heyningen (1942) reported that β-hemolysin can be obtained almost free from α-toxin at pH 7.5. General agreement was found in this experiment in that no α-hemolysin was detected when the final growth pH was 7.1 to 7.2. One factor involved in the lack of α-hemolysin production at this lower pH value may be related to the lack of chemical protection of the cells provided by a protein free medium. Again, in each case, whether or not glucose was added, the hemolysin yields per ml cell-N at 43°C were less than those at 37°C incubation.

Lecithinase and proteinase production. In most cases either no lecithinase or proteinase activity was detected or only trace activity was found. Strain BP6K (table 3) did show significant production of these two enzymes. The LV solution showed strong flocculation due to the release of free fatty acids and a marked color development from azocoll. An explanation for this one isolated case of lecithinase and proteinase production is not apparent. All of the cultures had been serially transferred numerous times in the synthetic medium so that the possibility of transferring trace amounts of a toxigenic ingredient from a non-synthetic medium can be eliminated. The important fact, however, is that these toxic fractions were produced in a chemically defined medium. Further investigation is in progress to determine toxigenic factors for lecithinase and proteinase.

In order to avoid precipitation of the phosphate buffer used in the defined medium, Wagner's (1949) LV solution was used in preference to...
other preparations which contain added calcium, (Oakley and Warrack, 1941; Macfarlane et al., 1941). Wagner's preparation contains no added calcium since as stated in his report, prolonged contact of the toxin and LV solution makes no difference in the amount of turbidity developed if trace amounts of calcium are present. That adequate trace calcium was present is shown by the fact that in the one case where marked turbidity developed, the reaction was apparent after an incubation period of only 30 min and no noticeable increase in turbidity occurred after refrigeration overnight.

The general acceptance of the identity of α-hemolysin and lecinthinase might possibly be questioned on the basis of finding, in many instances, high titers of α-hemolysin in the absence of detectable lecinthinase activity (Macfarlane et al., 1941; Macfarlane, 1950). MacLennan (1953) has indicated the possible identity of α-hemolysin and lecinthinase whose activity may differ from the lethal action of α-toxin due to the presence of two separate reactive groups on the same molecule. It is not inconceivable that manifestations of α-hemolysin and lecinthinase are also due to two separate reactive groups on the same molecule. However, proof as determined in these experiments is limited to the lack of quantitative agreement between α-hemolysin and lecinthinase production.

It is not too difficult to account for the production of toxins in a medium in which toxin production was not previously detected. Possible explanations could be based on differences in the quality of chemicals, to culture variations, and to various unknown factors. A more likely explanation, however, is based on differences in the temperature of incubation and on the age of the cultures at the time aliquots for toxin assays are taken. In designating the optimum temperature range for rapid growth as being 43 to 46°C, Boyd and co-workers apparently overlooked the possibility of the existence of multiple temperature optima and that the optimum temperature for rapid growth and that for toxin production may not be identical. Results of the present work show a marked reduction in toxin yields when cultures were incubated at 43°C as compared to 37°C. Boyd et al. (1948) also reported that maximum growth occurred between 16 and 20 hr of incubation. In this laboratory, hourly assays of toxin yields for the first 8 hr incubation of cultures has confirmed Gale's report that peak yields of these toxins occur between 4 and 7 hr of incubation. If Boyd et al. made toxin assays after 16 to 20 hr, they might have missed toxin detection since the activity of α- and θ-hemolysins has been found to decline rapidly after a 7 hr incubation period. In some experiments activity decreased several hundred per cent at 8 hr incubation from that found at 7 hr incubation for the same culture. After 24 hr of incubation no activity was found in the defined medium used.

Further work is in progress to investigate specific toxigenic factors for this group of organisms.

ACKNOWLEDGMENT

The author wishes to thank Mr. E. O. Hill of the University of Cincinnati College of Medicine for his cooperation in supplying cultures of strains BP6K and CN972.

SUMMARY

It has been shown that several strains of Clostridium perfringens have the capacity for producing α- and θ-hemolysins, proteinase and lecinthinase as shown by in vitro tests when the organisms are grown in a chemically defined medium.

Probable reasons for the lack of toxin production in an earlier investigation are discussed as being related to differences in the temperature of incubation and differences in the age of the cultures at the time of sampling for toxin assays.

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