Some Properties of Heat-Resistant and Heat-Sensitive Strains of *Clostridium perfringens*

I. Heat Resistance and Toxigenicity

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Received for publication 20 August 1966

ABSTRACT

Heat resistance at 100 C (D-values), sporulating ratios, toxigenicity for mice, and lecithinase activity (as micrograms per milliliter of enzyme, ascertained by the lecithovitellin reaction) were determined for four strains of *Clostridium perfringens*. A definite inverse relationship between thermal resistance and toxigenicity was found. The D-values ranged from 17.6 for the most heat-resistant strain to 0.3 for the strain possessing the least heat resistance, with corresponding lecithinase activities from 25 to 133 μg/ml of enzyme. The sporulating ratios did not differ greatly between the strains. The heat stability of the toxin was greater at 100 C than at 75 C. There was a noticeable difference between the heat stabilities of the toxin in the culture fluids of the heat-sensitive and heat-resistant strains at pH 7.0 when the toxic filtrates were held at 100 C. At a holding temperature of 75 C, a similar but lesser difference was observed at pH 5.5. Heat resistance and lecithinase activity did not change when a substrain of the least heat-resistant parent strain was obtained through heat selection by a single transfer, or when the most heat-resistant strain was transferred serially 12 times.

It has recently been reported that in various species of the genus *Clostridium* there may exist a definite relationship between sporulating potency, heat resistance, and toxigenicity (15, 16, 17, 20, 24). With special reference to *C. perfringens*, Yamagishi et al. (24) have shown that fewer toxicogenic strains were obtained when higher temperatures were employed during the isolation of the organism from soil.

The degree of resistance to thermal stress and the comparative quantity of α toxin production are two characteristics which have been used to differentiate between the classical infectious and the food-poisoning strains of *C. perfringens* (10). Other workers (9, 14, 23) do not hold to the view that only heat-resistant strains are capable of causing food poisoning; nevertheless, heat resistance and lecithinase activity are criteria by which different strains of *C. perfringens* appear to differ from one another.

The work reported here was undertaken to compare heat resistance and lecithinase activity of three strains of *C. perfringens* which had previously been isolated from sources associated with food-poisoning outbreaks with the same characteristics as demonstrated by an infectious strain. Lecithinase activity was accepted by the authors as a measure of α toxin production, and the two terms are used synonymously in this paper.

MATERIALS AND METHODS

Cultures. Four strains of *C. perfringens* were used: strain 26 no. 3624 type A of the American Type Culture Collection, a classical infectious strain; Hobb's serotype 2, National Collection of Type Cultures no. 8238, isolated from boiled beef suspected to have caused food poisoning; strain IU 1168, recovered by L. S. McClung from the feces of a patient suffering from food-poisoning symptoms; and strain T-65, isolated from turkey meat implicated in a food-poisoning outbreak in Madison, Wis.

Sporulation and heat resistance. The stock cultures were maintained frozen in cooked meat medium. For the heat resistance studies, the organisms were grown in Fluid Thioglycollate Medium (FTG; BBL) for 18 hr at 37 C. Transfers were then made into Thioglycollate Medium without Dextrose or Indicator (Difco), and, after 4 hr of incubation, this suspension was used to inoculate SEC broth (1) to give a 2.5% concentration of actively growing cultures. The sporulating medium was incubated for 24 hr at 37 C, and 

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and the total number of cells and spores was determined. Spore counts were obtained by plating the survivors after heating the cultures in SEC broth for 20 min at 75 C (6). SPS Agar (Difco) was used for all platings throughout the series of experiments.

The heat resistance of spores was determined on triplicate samples. Heating of 1-ml amounts of the spore suspension in SEC broth was done in soft glass tubes (4 by 150 mm) at 100 C for various periods of time after the contents of the tubes had been held at 75 C for 20 min to destroy the vegetative cells. D-values were computed from the straight-line portions of the thermal death time curves by use of the formula: 

\[ D = \frac{U}{\log b - \log a} \]

where \( D \) = time of exposure, \( b \) = initial spore population, and \( a \) = number of surviving spores per time of exposure.

In light of a question raised by Yamagishi et al. (24) regarding a proper definition of "heat-resistant" as applied to strains of C. perfringens, the present authors have expressed this characteristic as "sporulating ratio." The latter term denotes the ratio of total cell count to the "spore count" obtained after heating the SEC broth cultures for 20 min at 75 C.

Toxin production and lecithinase assay. The medium used was Noyes veal broth (1). It was inoculated to contain 2% of a 24-hr FTG culture, and incubated at 37 C. As soon as active growth and gas production became noticeable (from 2 to 4 hr), 4 ml of suspension was removed from the veal broth at hourly intervals and centrifuged at 27,000 X g for 5 min. The clear supernatant fluid was decanted and stored in a refrigerator. The lecithinase activity in vitro was determined by a slight modification of the method suggested by Elner (5). A 0.3-ml amount of the toxic culture fluids was routinely used for assaying. Borate-calcium-gelatin-saline diluent was added to give a volume of 1 ml. A 2-ml amount of egg yolk substrate was added, and the reaction mixture was incubated at 42 C for 60 min.

A blank was prepared for each unknown. Before the addition of the egg yolk suspension, 0.05 ml of undiluted antitoxin (Sigma Chemical Co., St. Louis, Mo.) was added to the same volume of supernatant fluid that was used as sample. After incubation, the volume of the blanks was adjusted with 9.95 ml of saline, whereas 10 ml of saline containing 0.05 ml of antitoxin was added to each sample. The optical densities were read at 815 m\(\mu\) instead of 590 m\(\mu\) (Orlowska et al., Proc. Intern. Congr. Biochem., 4th, Vienna, 1958, p. 207, 1959). A standard curve was prepared from a series of standard solutions containing from 0 to 40 \(\mu\)g of standard lecithinase (\(\alpha\) toxin, Sigma Chemical Co.) in the diluent (Fig. 1).

Animal procedures. The minimal lethal dose for mice was compared with the lecithinase activity in veal broth of each of the four strains. Graded doses of the standard toxin (in micrograms) were injected intraperitoneally into Swiss mice weighing 15 to 20 g. The smallest amount of toxin killing five out of five animals was considered the minimal lethal dose (MLD).

The toxicity of the supernatant fluids of the four test strains was similarly determined. Appropriate dilutions of the toxic culture fluids in 1-ml volumes were used for the injections. Five mice were used per dilution. The smallest volume of supernatant fluid present in 1 ml of injection that would kill all five animals within 48 hr was considered the MLD.

RESULTS AND DISCUSSION

The sporulating ratios (Table 1) did not differ widely among the four strains; however, there were great differences in heat resistance at 100 C, expressed in D-values and shown by the thermal death time curves in Fig. 2. The D-values ranged from 0.31 and 0.39 for strain ATCC 3624, indicating high heat sensitivity, to 13.0 and 17.6 for the very heat-resistant strain NCTC 8238. Strain IU-1168 had very low D-values (1.0, 1.5); however, a few spores always survived heating for 30 min, as evidenced by growth in FTG subsequent to inoculation by the heated spore suspension. With strain ATCC 3624, survivors could never be demonstrated after 5 min of heating at 100 C. Some spores of strain NCTC 8238 consistently survived heating for 120 min, and, with strain T-65, survivors could always be demonstrated after culturing in FTG after 60 min of heating. The counts for cells recoverable on SPS Agar from the sporulating medium held at 100 C for the maximal length of time which still permitted subsequent growth in FTG were always in the range from 5 to 30 spores per milliliter. This indicated that the observed maximal heat toler-
TABLE 1. Sporulating ratios and D-values of four strains of Clostridium perfringens

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of viable cells before heating (per ml)</th>
<th>No. of spores surviving after heating* (per ml)</th>
<th>Ratio</th>
<th>D-valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 3624</td>
<td>4.2 \times 10^6</td>
<td>4.5 \times 10^4</td>
<td>0.11</td>
<td>0.31</td>
</tr>
<tr>
<td>IU-1168</td>
<td>6.1 \times 10^6</td>
<td>6.5 \times 10^4</td>
<td>0.11</td>
<td>0.39</td>
</tr>
<tr>
<td>T-65</td>
<td>2.7 \times 10^6</td>
<td>4.0 \times 10^5</td>
<td>0.15</td>
<td>1.0</td>
</tr>
<tr>
<td>NCTC 8238</td>
<td>5.9 \times 10^6</td>
<td>1.2 \times 10^6</td>
<td>0.20</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Held at 75 C for 20 min.

Strains ATCC 3624 and IU-1168 with average D-values of 0.35 and 1.25, respectively, produced 5.3 and 2.4 times as much lecithinase as did strains NCTC 8238 and T-65 with average D-values of 15.3 and 5.9, respectively.

The results obtained for strain IU-1168 do not support the classical definition of a food-poison-
ing strain of *C. perfringens*. The relatively low D-values but high toxigenicity of this strain set it apart from strains NCTC 8238 and T-65, which exhibited the typical characteristics of a food-poisoning strain—high heat resistance but low α toxin production; however, various investigators (3, 9, 23) have suggested that possibly any strain of *C. perfringens* regardless of its heat resistance can cause food-poisoning symptoms.

The question of the relationship of the toxin to gastrointestinal disturbance in the human being remains open. Nygren (18) has suggested that phosphorylcholine, the end product of the action of the α toxin (lecithinase C) on lecithin, is the causative factor of the intestinal disturbances. He based his postulate on observations of mice—and one monkey—whose intestinal passage times were dramatically reduced when they were fed 1-mg amounts of phosphorylcholine. Dack (4) reiterated the viewpoint that, in naturally occurring food-poisoning outbreaks and in feeding experiments involving *C. perfringens*, large numbers of living organisms must be present to cause food-poisoning symptoms. This view does not, of course, exclude the possibility of lecithinase production by the organisms in the gastrointestinal tract. However, Weiss et al. (22) have been unsuccessful in demonstrating a definite relationship between the occurrence of gastrointestinal symptoms in rhesus monkeys and their ingestion of viable cells, purified lecithinase, or phosphorylcholine.

Current investigations are being done in this laboratory to determine whether lecithinase activity can be detected in the gastrointestinal tract of rats which have been fed viable cell loads of varying magnitudes of different strains of *C. perfringens*. Attempts are also being made to determine the presence of a possible α toxin neutralizing substance (7) in the gastrointestinal tract of rats.

To obtain a more definite relationship between lecithinase activity and toxigenicity than that based on arbitrarily defined units (11, 13, 21), the MLD of the α toxin was determined in terms of weight. There was good agreement between the MLD for mice in terms of the amounts of lecithinase determined in the culture fluids, and in terms of weight of purified lecithinase, which was found to be 20 μg.

At 75°C, the heat stability of the enzyme in the culture fluids of the four strains was greater at pH 5.5 than at pH 7.0, except for strain IU-1168 which, after heating, showed marked lecithinase activity at the higher pH (Table 3). In the supernatant fluids of the two most heat-resistant strains, T-65 and NCTC 8238, at pH 5.5 and after heating, a higher percentage activity was observed than in the supernatant fluids of the two least heat-resistant strains. This is all the more noteworthy, because the activities of these two strains before heating were only 61.6 and 27.0%, respectively, of the highest activity produced by strain IU-1168. The comparable activity for strain ATCC 3624 was 98.6%.

Heating the toxic filtrates at 100°C produced quite different results. The activities for strains ATCC 3624 and IU-1168 remaining after heating were higher at pH 5.5 than at pH 7.0. The reverse was true for strain T-65, and especially for strain NCTC 8238, the toxin of which exhibited a remarkable heat stability in the supernatant fluid at pH 7.0. It surpassed even that of the standard lecithinase which, in all other cases, was much more heat-stable than the active principle in the culture fluids. This is in contrast to the findings of Kushner (12), who noted that the lecithinase activity in his purified toxin was almost completely destroyed after exposure to 100°C for 5 min. Kushner reported as a curious finding that heating the filtrate for 5 min at 60°C destroyed more activity than did heating for the same time at 100°C, although anomalous heat inactivation of *C. perfringens* lecithinase and of *Bacillus cereus*, as well as of other enzymes, has been known for many years. Guillaumie et al. (8) reported that, in an acidic or slightly alkaline medium, the activities of freshly prepared *C. perfringens* toxin were suppressed after heating for 5 min at 70°C, but not at 100°C. Exposure to 100°C subsequent to heating at 70°C partially restored the lethal, hemolytic, and necrotic action of the toxin.

Chu (2) made similar observations about the effect of heating on *B. cereus* hemolysins, the activity of which was completely destroyed after 10 min at 60°C, whereas boiling for 10 min only destroyed 80% of its activity. This investigator did not, however, find an anomalous heat inactivation with either *C. perfringens* or *B. cereus* lecithinase.

Smith and Gardner (79) investigated some of the factors affecting the irregular heat inactivation of lecithinase and concluded that formation of a
complex by interaction of lecithinase with calcium or magnesium ions takes place at 65°C. This complex was believed to be dissociated at 100°C, followed by the formation of an insoluble calcium phosphate salt and the subsequent release of the enzyme.

In the present study, the residual lecithinase activity observed in the culture fluids after heating at 100 or 75°C was probably dictated by the type of reactions defined by Smith and Gardner (19). The results reported here were not consistent; nor, in some cases, were the differences at the various time periods marked. One might speculate that these results represented: (i) a possible interaction between varying quantities of lecithinase, (ii) the effect of pH or the complexing of the enzyme with the metal ions, (iii) the solubility of the calcium or magnesium phosphate compounds formed, or a combination of these factors.

No decrease in lecithinase activity was observed when the most heat-resistant spores of strain ATCC 3624 surviving heating at 100°C for 5 min were subcultured for toxin production, and the heat resistance of the substrain had not increased (Table 4).

Conversely, strain NCTC 8238 had not lost its high heat resistance after 12 daily transfers in FTG, and the lecithinase activity was unaffected (Table 4).

### Table 3. Heat stability of lecithinase

<table>
<thead>
<tr>
<th>Holding temp</th>
<th>pH</th>
<th>Source of lecithinase</th>
<th>OD at 815 μM (zero-time)</th>
<th>Per cent activity remaining after heating for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>C 75</td>
<td>7.0</td>
<td>Standard (40 μg)</td>
<td>0.848</td>
<td>65.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATCC 3624</td>
<td>1.22</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IU-1168</td>
<td>1.39</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T-65</td>
<td>0.396</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCTC 8238</td>
<td>0.214</td>
<td>0</td>
</tr>
<tr>
<td>100 5.5</td>
<td>7.0</td>
<td>Standard</td>
<td>0.800</td>
<td>43.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATCC 3624</td>
<td>1.40</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IU-1168</td>
<td>1.42</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T-65</td>
<td>0.875</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCTC 8238</td>
<td>0.380</td>
<td>11.8</td>
</tr>
<tr>
<td>5.5</td>
<td>7.0</td>
<td>Standard</td>
<td>0.865</td>
<td>88.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATCC 3624</td>
<td>1.10</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IU-1168</td>
<td>1.40</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T-65</td>
<td>0.360</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCTC 8238</td>
<td>0.268</td>
<td>100</td>
</tr>
</tbody>
</table>

a Lecithinase, in 1-ml amounts, was heated in screw-cap test tubes. Results are mean values of at least four determinations.
b Standard lecithinase was determined in diluent, and lecithinase from the indicated strains, in culture fluid.

### Table 4. Effect of subculturing on heat resistance and lecithinase activity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Heat resistance at 100°C</th>
<th>Lecithinase activity (OD)</th>
<th>Heat resistance at 100°C</th>
<th>Lecithinase activity (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td></td>
<td>min</td>
<td></td>
</tr>
<tr>
<td>ATCC-3624</td>
<td>5</td>
<td>1.5</td>
<td>5a</td>
<td>1.5</td>
</tr>
<tr>
<td>NCTC-8238</td>
<td>120</td>
<td>0.285</td>
<td>120b</td>
<td>0.265</td>
</tr>
</tbody>
</table>

a Heat-selected strain obtained by single subculturing of surviving organisms of parent strain after heating at 100°C for 5 min.
b Substrain obtained from parent strain by 12 successive transfers.

Apparent low heat resistance could be induced into spores of strain NCTC 8238 by use of Ellner's medium (9); however, when these spores were used as inoculum for toxin production in veal broth, the maximal lecithinase activity was practically unaffected. Likewise, when the vegetative cells from the veal broth were allowed to sporulate in SEC broth, the spores were again as heat-resistant as those produced in SEC broth directly.

These results lend support to the view that the inverse relationship between thermostability and
lecithinase activity for *C. perfringens* is stable, even though the apparent heat resistance may be affected by environmental conditions.

**ACKNOWLEDGMENT**

This investigation was supported by U.S. Department of Health, Education, and Welfare research grant EF 00131-6 from the Bureau of State Service, Division of Environmental Engineering and Food Protection.

**LITERATURE CITED**

2. **Chu, H. P.** 1949. The lecithinase of *Bacillus cereus* and its comparison with *Clostridium welchii* α toxin. J. Gen. Microbiol. 3:255-273.