THE RELATIONSHIP OF TOXIC FRACTIONS OF A FILTRATE OF CLOSTRIDIUM PERFRINGENS TYPE A TO THE PATHOGENESIS OF CLOSTRIDIAL MYONECROSIS*

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Considerable progress has been made in defining toxins elaborated by Clostridium perfringens. Although the nature of the theta toxin is not known, the majority of the toxins have been shown to be enzymatic: lecithinase, or alpha toxin, collagenase, hyaluronidase, and desoxyribonuclease. Nevertheless, despite numerous and excellent descriptions of the disease, comparatively little progress has been made in correlating in vitro activity of the toxins with the signs and symptoms seen in clostridial myonecrosis. It therefore seemed desirable to study a toxic filtrate of Cl. perfringens, using features of the clinical picture as criteria of its activity, with the hope that such a correlation might be made.

Study of case histories of patients with gas gangrene shows that cardiovascular collapse is one of the early and constant signs in this disease. Moreover, pathological studies of the local lesion reveal the absence of leucocytic infiltration and phagocytosis. The present work was undertaken to determine whether there is a significant relationship between these features of the clinical picture and the in vitro activity of Cl. perfringens toxins.

Materials and Methods

The organism used in these studies was Cl. perfringens type A, strain 150.1 A toxic filtrate was prepared from a 6 to 8 hour broth culture of this organism using the procedure described by Kull (1).

The method of alpha toxin determination was essentially that used by Owen and associates (2), and the theta toxin was assayed by a hemolytic technic described by van Heyningen using rabbit erythrocytes (3). Combined hemolytic activity (alpha plus theta) was determined by the method of van Heyningen (3).

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1 Obtained through the courtesy of Dr. L. S. McClung, Indiana University, Bloomington.
Human synovial fluid was used as a substrate in measuring hyaluronidase activity. This determination as well as that of collagenase activity followed the methods described by Oakley (4, 5). The LD₅₀ for mice was calculated from the results of intravenous injection.

The technic used for the measurement of epinephrine sensitivity of the peripheral circulation of the rat was fundamentally the same as that described by Zweifach (6-8). Female rats of the Sprague-Dawley strain were anesthetized by intramuscular injection of 4 mg. of secobarbital sodium (Lilly) per 100 gm. body weight. The meso-appendix of the anesthetized animal was exteriorized, draped over a lucite block, and flooded with warm Ringer-gelatin solution to maintain the tissue at approximately 37°C. After microscopic observation of normal rate of flow through metarterioles, capillaries, and venules, one such terminal vascular bed unit was selected for an epinephrine sensitivity study. The highest dilution of epinephrine capable of producing a “threshold constrictor response” was determined. This response was defined as a temporary constriction just sufficient to stop blood flow through the capillaries. In order to avoid additive response of the tissue to epinephrine, a lapse of 3 minutes was allowed between tests.

Phagocytosis inhibition was determined by the method of Hamburger (9) using rat leukocytes and heat-killed B. anthracis as a test organism. The leukocytes were obtained by stimulating the production of peritoneal exudate as described by Nungester and Ames (10). The

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2 Obtained through the courtesy of Dr. I. Duff, University of Michigan Hospital, Ann Arbor.

3 0.3% Difco gelatin in Ringer solution.
The heavy metal-alcohol method, introduced by Cohn and associates in 1950 (11) for serum fractionation, was modified to suit the needs of this investigation in fractionating the crude toxic filtrate of *Clostridium perfringens*. Except for the separation of fraction R3A1, Cohn's method 10 was followed in all essential points (see Chart 1). In fractionating R3 the following changes were made. The ethanol-acetate mixture (pH 4.1, ionic strength 0.8) was slowly added to the toxin with continuous stirring at $-5^\circ C$. This mixture then was stirred for 1.5 to 2 hours at $-20^\circ C$, at which time a light precipitate appeared. After storing the material overnight at $-25^\circ C$, it was centrifuged at 1800 r.f.m. (180 g) for 1 hour at $-10^\circ C$. The precipitate thus obtained, designated as R3A1, was found to be soluble at $-5^\circ C$.

### RESULTS

#### The Demonstration of Recognized Toxins in the Crude Filtrate and Its Various Fractions

Aliquots (25 ml.) of the crude filtrate (R3) of *Clostridium perfringens* were dialyzed for 24 hours against running tap water in the cold (10°C.) and then dried by the lyophil process. This dried material was dissolved in 0.9 per cent NaCl and characterized in terms of its alpha toxin, theta toxin, collagenase, and hyaluronidase activities. The purpose of this characterization was twofold: (a) to determine whether the filtrate used in this investigation had all the essential properties of a type A toxin and to determine the relative potency of the filtrate in terms of *Clostridium perfringens* toxins described in the literature; (b) to have a standard preparation on which to base the progress of future fractionation.

As seen from the data presented in Table I, the crude material contained all the components described for type A filtrates, for which tests were made. The concentrations, however, did not prove to be particularly high. These toxic components could be neutralized by specific *Clostridium perfringens* antiserums.4

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**TABLE I**

Characterization of *Clostridium perfringens* Filtrate (R3) in Terms of Recognized Toxins

<table>
<thead>
<tr>
<th>Toxic activity</th>
<th>Smallest amount giving positive reaction</th>
<th>50 per cent end-point mg.</th>
<th>Units/mg.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha</td>
<td>0.37</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Alpha + theta</td>
<td></td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Theta</td>
<td>0.07</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>0.06</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Collagenase</td>
<td>0.25</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>LD$\text{mg}$/mg†</td>
<td>—</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

* One unit is the smallest amount of toxin, expressed in milligrams, that will give a positive reaction.

† The figures in this table represent the average of a minimum of three tests.

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4 Obtained through the courtesy of Parke, Davis & Co., Detroit, and Dr. J. D. MacLennan, Columbia University, New York.
The filtrate was then fractionated as previously described and the several components were tested for toxin activity.

On examination of Table II it is seen that alpha, theta, and hyaluronidase activities were considerably increased in fraction R3A1. A fifty-fold increase in the LD₅₀ titer was also noted with this fraction.

In fraction R3F2 alpha toxin activity was increased to 45 units as compared with 2.7 units in the crude material. Collagenase activity was increased to 50 units from a previous 4 units, the theta toxin to 50 units from a previous 7 units, and hyaluronidase to 100 units from a previous 16 units.

When the crude filtrate and its various fractions had been characterized in terms of recognized toxins, as outlined above, the study was directed toward an evaluation of the relationship of these products to actions more directly associated with the pathogenesis of gas gangrene, viz. cardiovascular collapse and absence of phagocytosis.

Studies of the Peripheral Circulation

Rats were prepared as previously described, control epinephrine thresholds were established, and only those animals were used in which this threshold fell within the accepted normal range (1 × 10⁻⁶ and 6 × 10⁻⁸ dilutions). Several experiments were performed to ascertain how long the meso-appendix could be stimulated with threshold doses of epinephrine without changing the sensitivity of the peripheral vessels. If normal animals were used, only minimal changes in sensitivity were seen during the 1st hour of stimulation with epinephrine. An increase from 3 × 10⁻⁶ to 6 × 10⁻⁶ mg. occasionally occurred. These results confirmed observations made on some thirty animals by one of us (D.F.B.) using the same standard experimental conditions.

After establishing the normal vascular sensitivity, 6 mg. of toxin per 100 gm. of body weight was injected intramuscularly. The vascular response to epinephrine was then tested at 3 to 5 minute intervals. After 45 minutes, it was found that an animal previously responding to a concentration 3 × 10⁻⁶ of epinephrine, but not to 6 × 10⁻⁶, would now respond to a concentration of 3 × 10⁻⁷, giving a tenfold increase in sensitivity. The test with this crude material was repeated four times, showing an average tenfold increase in sensitivity.

To test the specificity of the reaction, 6 mg. of toxin in 0.4 ml. Ringer-gelatin solution was incubated for 30 minutes at 25°C. with 0.01 ml. of commercial antitoxin. This toxin-antitoxin mixture was then injected intramuscularly and the test repeated as previously described.

The specific antitoxin was shown to prevent the increase in vascular sensitivity during a 45 minute period of observation. Subsequent experiments showed that normal horse serum would not inhibit the reaction of the toxin.

Having established the specificity of the reaction, the various fractions were

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* Aqueous epinephrine 1:1000 (10⁻⁹) was used.
tested for reactivity by this procedure. Fraction R3B1 was shown to give an 86-fold increase in sensitivity compared to the tenfold increase for the crude material (Table II). Further fractionation of R3B1 resulted in a decrease in activity. The above data are based on an average of six or more tests. Although the results were reproducible only within wide limits, weak, moderate, and strong reactions could easily be recognized. An attempt to improve quantitation by intravenous injection of toxin was not successful.

**TABLE II**

Comparison of Circulation and Phagocytosis Inhibition Factors with Other Activities of Fractions of *C. perfringens* Filtrate

<table>
<thead>
<tr>
<th>Toxic fraction</th>
<th>Alpha <em>units/mg.</em></th>
<th>Theta <em>units/mg.</em></th>
<th>Hyaluronidase <em>units/mg.</em></th>
<th>Collagenase <em>units/mg.</em></th>
<th>Per cent phagocytosis test</th>
<th>Per cent phagocytosis control</th>
<th>Circulation threshold</th>
<th>Initial threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3</td>
<td>3</td>
<td>7</td>
<td>16</td>
<td>4</td>
<td>0.38</td>
<td>10</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>R3A1</td>
<td>25</td>
<td>66</td>
<td>133</td>
<td>4</td>
<td>0.56</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>R3A2</td>
<td>1</td>
<td>1</td>
<td>33</td>
<td>2</td>
<td>0.48</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>R3B1</td>
<td>1</td>
<td>3</td>
<td>25</td>
<td>2</td>
<td>0.41</td>
<td>86</td>
<td>86</td>
<td>86</td>
</tr>
<tr>
<td>R3C1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0.49</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R3C2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.51</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R3D1</td>
<td>7</td>
<td>2</td>
<td>91</td>
<td>4</td>
<td>1.0</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>R3D2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.96</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>R3E1</td>
<td>11</td>
<td>2</td>
<td>55</td>
<td>8</td>
<td>0.99</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R3E2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1.0</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>R3F1</td>
<td>5</td>
<td>10</td>
<td>16</td>
<td>4</td>
<td>0.55</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>R3F2</td>
<td>45</td>
<td>50</td>
<td>100</td>
<td>50</td>
<td>0.33</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

* Represents average of at least 3 tests.
† Reaction elicited by injecting 6 mg./100 gm. body weight.
§ Represents average of 2 to 6 tests.
||Highest value.

Examination of Table II reveals no correlation between amounts of known toxins per fraction and an effect on circulation after intramuscular injection. It is seen that 6 mg. of R3A1, although containing 150 units of alpha toxin, 396 units of theta toxin, 798 units of hyaluronidase, and 24 units of collagenase, gave only a fourfold increase in sensitivity to epinephrine; while 6 mg. of R3B1, containing only 6 units of alpha toxin, 9 units of theta toxin, 150 units of hyaluronidase, and 12 units of collagenase, gave an 86-fold increase.

Furthermore, it should be noted that fraction R3D2, having no alpha toxin, theta toxin, hyaluronidase, or collagenase, gave a sixfold increase in epinephrine sensitivity. When samples (10 mg. per ml.) of crude toxin and fraction R3B1 were heated at 56°C. for 1 hour, there was a complete loss of their ability to potentiate the response to epinephrine. In this respect the potentiating sub-
stance or “circulation factor” differed from the alpha toxin which is stable at 56°C. Theta toxin is also destroyed at this temperature.

In hemorrhagic shock, the early compensatory reaction which can be detected by a sharp increase in the sensitivity of peripheral vessels to epinephrine, has been credited by Zweifach (8) to the action of a renal factor. In view of this fact it seemed of interest to investigate the possible role of the kidney in the reaction of the rat to Cl. perfringens toxin. For this purpose, rats were lightly anesthetized with 2.5 to 3 mg. of secenal sodium per 100 gm. of body weight. With a minimum of trauma the kidneys were removed by a dorsal incision. The animals responding inside the normal range (1 × 10^{-4} and 6 × 10^{-4}) following the nephrectomy were used. On examination of Table III it is seen that nephrectomy diminished vascular response to the epinephrine after toxin injection. In the unnephrectomized animal there was an 86-fold average increase in vascular sensitivity, while there was only a thirteen-fold increase in the nephrectomized animal.

The importance of the adrenal gland in situations of stress is well known. Thus, attempts were made to elucidate the possible role of the adrenals in the present reaction. Rats were lightly anesthetized and the adrenals removed. The animals were allowed to recover from the operation and after 3 to 4 hours were again anesthetized; the normal end-point was established and the reaction of the animals to 6 mg. of toxin per 100 gm. body weight was determined. The normal threshold range of these animals (0.5 × 10^{-6} to 3 × 10^{-6}) was slightly lower than that of the controls. Using R3B1, a 64-fold increase in sensitivity was obtained, compared with an average 86-fold increase in the non-operated animal (Table III).

### Table III

<table>
<thead>
<tr>
<th></th>
<th>Response normal animals*</th>
<th>Response nephrectomized animals*</th>
<th>Response adrenalectomized animals*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Means</td>
<td>86.7 †</td>
<td>13.5</td>
<td>64.2</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>45.0</td>
<td>12.8</td>
<td>18.6</td>
</tr>
<tr>
<td>Standard error of mean</td>
<td>18.4</td>
<td>5.2</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Comparisons:
- Normal vs. nephrectomized ..................... $p < 0.01$ (significant).
- Normal vs. adrenalectomized ................... $p > 0.10$ (not significant).
- Nephrectomized vs. adrenalectomized ........... $p < 0.001$ (significant).

* Six tests.
† Initial Threshold
‡ Final Threshold
Phagocytosis Studies

A study of the local lesion of clostridial myonecrosis reveals that little if any phagocytosis has occurred (12). In view of this observation and the possible importance of phagocytosis as a normal defense mechanism of the host, studies were undertaken to elucidate the effect of Cl. perfringens toxins on phagocytosis by polymorphonuclear leucocytes.

The concentration of toxin used in this test was 1 mg. per ml. and results were expressed as per cent phagocytosis in test system. If no inhibition occurred per cent phagocytosis in control.

<table>
<thead>
<tr>
<th>Amount crude toxin</th>
<th>Serum</th>
<th>Phagocytosis</th>
<th>Ratio Test/Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td></td>
<td>Per cent</td>
<td>Average</td>
</tr>
<tr>
<td>1</td>
<td>0.005 ml. antitoxin*</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td>1</td>
<td>0.005 ml. antitoxin†</td>
<td>28</td>
<td>14</td>
</tr>
<tr>
<td>1</td>
<td>0.005 ml. normal horse</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>1</td>
<td>0.005 ml. antitoxin*</td>
<td>25</td>
<td>11</td>
</tr>
</tbody>
</table>

* Antiserum against fraction R3C2.
† Commercial polyvalent antiserum.

From the results shown in Table IV it is seen that the crude filtrate partially inhibited phagocytosis (ratio 0.38). This inhibition could be prevented by the addition of specific antitoxic serums, while normal horse serum failed to neutralize inhibitory effects. When the specificity of the reaction had been established, the various fractions were tested by this procedure. From Table II it will be seen that the power to inhibit phagocytosis was maintained until R3B1 was precipitated with the barium acetate–alcohol solution. Fractions R3D1, R3D2, R3E1, and R3E2 showed little if any inhibitory activity.

On examination of Table II, it will also be noted that fractions R3C1 and R3C2 do not show activity of any of the known toxins, with the exception of...
collagenase in R3C1, though both possess the power to inhibit phagocytosis. This action of R3C2 could be inhibited by specific commercial antitoxins and by antiserums prepared against several of the fractions. It should further be noted that fractions R3D1 and R3E1, though showing no ability to inhibit phagocytosis, contain more alpha toxin and hyaluronidase than the crude material. The factor responsible for inhibition of phagocytosis is resistant to heating for 1 hour at 56°C. This would further indicate that the factor is separate from the theta toxin, which is heat-labile. To obtain more specific data on the relationship between this phagocytosis-inhibition factor and the known toxins, immunological studies were performed. Antitoxin against a partially purified fraction, R3C2, was prepared. Using 1 mg. of dried, crude filtrate (R3) in the system, the phagocytosis-inhibition reaction could be prevented by 0.005 ml. of R3C2 antitoxin, but theta toxin, alpha toxin, collagenase, and hyaluronidase activities could not be blocked by any concentration of the antiserum.

**DISCUSSION**

Careful studies of case histories of patients with clostridial myonecrosis (13) have demonstrated that cardiovascular collapse is an almost constant characteristic, yet few studies on this feature of the pathological physiology of gas gangrene have been made.

The syndrome when fully developed is characterized by a high pulse rate which is frequently dicrotic. The systolic and diastolic blood pressures are low. The patient's skin is pale, cold, and clammy. Veins are often collapsed, and the patient does not appear to respond to conventional shock treatment.

Noting the severe vascular changes in gas gangrene, it was felt that additional information could be obtained by direct microscopic observation of the peripheral vascular bed. Since gas gangrene is essentially a disease of muscle, intramuscular injection was used as the route of choice for testing the toxic activities of the filtrate. Following the injection of fraction R3B1 (6 mg. per 100 gm.) the epinephrine sensitivity of the peripheral vessels of the mesoappendix was greatly increased (86-fold). This activity of the toxin could be inhibited by the use of specific antitoxic serums. An increase in epinephrine sensitivity was also obtained after intravenous injection of the toxin. The response was gradual rather than immediate. Zweifach (7), studying the effect of a variety of substances on the peripheral vascular bed, injected, intravenously, 0.05 mg. of *Clostridium perfringens* toxin into 100 gm. rats. No change in the epinephrine response was seen. Using dogs, he injected sufficient concentrations of toxins to produce a lethal effect in 3 to 5 hours. For several hours, he observed no change, then capillary damage was seen, which was accompanied by a hyper-reactive stage. In the present study the amount of *Clostridium perfringens* toxin was about twelve times that used by Zweifach, which in part may explain the difference in results.
Some studies on the mechanism of the observed epinephrine-potentiating effects were made. Since Zweifach (8) previously had demonstrated the presence of a vaso-excitor material (VEM) in the kidney after graded hemorrhage, it was of interest to see whether \textit{Cl. perfringens} toxins would act directly on the blood vessels, or whether the increase of the epinephrine sensitivity was mediated by the kidney, as occurs in hemorrhagic and traumatic shock. Bilateral nephrectomy greatly reduced but did not completely abolish the response of the capillary bed of the animals to toxin (R3B1). Normal animals responded with an 86-fold increase, while the nephrectomized animals gave only a 13-fold increase in sensitivity to epinephrine. This suggests that the kidney plays an important role in the potentiating effect observed. It also tends to place the animals' response to \textit{Cl. perfringens} toxins in the general framework seen in hemorrhagic and other types of shock. It would be of great interest to follow the reaction for several hours and see whether vaso-depressor material (VDM) is produced in the final stages, thus completing the typical sequence as reported in fatal experimental shock (6-8).

The importance of the adrenals, both the medulla and the cortex, in situations of stress is well known. For this reason the effect of bilateral adrenalectomy on the circulatory response was tested. The response of adrenalectomized animals was a 64-fold increase in epinephrine sensitivity, as compared to an 86-fold increase in normal animals, suggesting that the adrenals are not vital in this phase of the circulatory response to \textit{Cl. perfringens} toxins.

The experimental data showed no apparent relationship between the previously described toxins and the circulatory factor. Fractions showing high alpha toxin, theta toxin, hyaluronidase, and collagenase activities potentiated the epinephrine sensitivity of the peripheral vessels only slightly, while fractions containing very small amounts of these recognized toxins may have caused a severe hyper-reactivity. Furthermore, the circulation response could be elicited from fractions showing none of the above activities. The circulation factor is heat-labile; the only other heat-labile component previously described is the theta toxin. The latter is present in very low concentrations in fraction R3B1 which gives an 86-fold increase in epinephrine sensitivity.

The above observations, though not conclusive, tend to indicate that this "circulation factor" is independent of either the alpha or theta toxins. The significance of this hyper-reactive state of the meso-appendix capillary bed in either the shock syndrome or clostridial myonecrosis is unknown. An interpretation will have to await further basic research in capillary physiology. It is of considerable interest, however, that a substance seemingly independent of the alpha toxin, acts on the peripheral circulation simulating a response seen in both human beings and experimental animals during shock.

Since the early work of McNee and Dunn (12), the conspicuous absence of leucocytic infiltration into the gas gangrene lesion has been recognized. Furthermore, polymorphonuclear leucocytes present in the lesion are generally
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free of organisms, indicating that little if any phagocytosis has taken place. In view of these observations, and of the possible importance of phagocytosis as a defense mechanism of the host, studies were undertaken to elucidate the effect of Cl. perfringens toxins on phagocytosis by polymorphonuclear leukocytes. Under the conditions of the experiments, phagocytosis of heat-killed Bacillus anthracis could be inhibited to the extent of 44 to 67 per cent by the crude toxin and most of its fractions. This inhibition of phagocytosis could be prevented by the use of specific Cl. perfringens antitoxins. A fraction, R3C2, was isolated from a crude filtrate of Cl. perfringens culture which would inhibit phagocytosis, but failed to give any alpha toxin, theta toxin, hyaluronidase, or collagenase reaction. An antiserum prepared against this heat-stable compound did not inhibit the theta toxin, alpha toxin, or collagenase activities in a dilution of 1:10, but prevented the inhibition of phagocytosis at a dilution of 1:5000. Commercial antitoxin (0.005 ml.) would block all the above toxic activities.

From the above observations, it is seen that a partially purified substance related to phagocytosis inhibition has been isolated from filtrates of Cl. perfringens. This material does not appear to correspond to any of the fractions previously described in the literature.

Butler (14), working with recently isolated strains of Cl. perfringens from postabortal infections, noted that heavily encapsulated organisms would resist phagocytosis by human leucocytes. The use of antitoxic serums usually resulted in but little increase in the number of bacilli phagocytized, while the use of antibacterial serums had a striking effect. These results seemingly contradict the data obtained in this study; however, two completely different systems were studied. Butler studied the effect of Cl. perfringens capsular material on phagocytosis, while in this investigation, the effect of exotoxins on phagocytosis was tested. It might, therefore, be postulated that in infection either or both factors contribute to the invasiveness of the organism.

SUMMARY

A crude filtrate from a culture of Clostridium perfringens, type A, was fractionated by a heavy metal-alcohol technique, and some degree of concentration of biologically active factors was achieved. Both the crude material and the fractions obtained were characterized in terms of their alpha toxin, theta toxin, hyaluronidase, and collagenase activities.

The filtrate and fractions were tested for their effect on the peripheral circulation of the rat, using the epinephrine threshold technique. The crude material and several fractions caused a sharp increase in epinephrine sensitivity of the capillary bed of the meso-appendix of the rat; fraction R3B1 giving an 86-fold increase in sensitivity. This reaction could be inhibited by specific antitoxic serums but not by normal serum. The “circulation factor” was shown
to be heat-labile and appears to be independent of either the alpha or theta toxins. Bilateral nephrectomy greatly reduced, but did not abolish, the effect of the toxin, while the threshold response to epinephrine was not materially changed following bilateral adrenalectomy.

The crude filtrate and several fractions were shown to inhibit the phagocytosis of heat-killed *B. anthracis* to the extent of 40 to 50 per cent. Fraction R3C2 was devoid of all biological properties studied here, except phagocytosis inhibition, suggesting that the factor responsible for this activity is distinct from the "classical" toxins and the "circulation factor." Moreover, a 1:5000 dilution of an antiserum prepared against this fraction would completely neutralize the phagocytosis inhibition factor but failed to inhibit any of the other toxic activities.

Since cardiovascular collapse and absence of phagocytosis are two significant clinical findings in gas gangrene, the possible roles of the "circulation" and "phagocytosis inhibition" factors in the pathogenesis of this disease are discussed.

**BIBLIOGRAPHY**