INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeeb Road
Ann Arbor, Michigan 48106
PARK, Yoong, 1944-
QUANTIFICATION AND SELECTED PROPERTIES
OF ALPHA TOXIN OF CLOSTRIDIUM PERFRINGENS.

The Ohio State University, Ph.D., 1976
Food Technology

*Xerox University Microfilms*, Ann Arbor, Michigan 48106
QUANTIFICATION AND SELECTED PROPERTIES OF 
ALPHA TOXIN OF CLOSTRIDIUM PERFRINGENS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for 
the Degree Doctor of Philosophy in the Graduate 
School of The Ohio State University

By

Yoong Park, B.Sc., M.Sc.

The Ohio State University
1976

Reading Committee:

J. R. Chipley
M. Mangino
T. Kristoffersen
E. M. Mikolajcik

Approved By

E. M. Mikolajcik
Advisor
Department of Food Science 
and Nutrition
ACKNOWLEDGMENTS

I wish to express my sincere appreciation and deepest gratitude to my advisor, Professor E. M. Mikolajcik, for his patience and encouragement throughout my graduate program, and for his guidance and assistance during the research and in the preparation of the manuscript; to Professor T. Kristoffersen, Chairman, Department of Food Science and Nutrition, for providing the opportunity for graduate study; and to Professor W. J. Harper and other faculty members of the department for their cooperation and help in my training program.

Special thanks are extended to Professor A. C. Ottolenghi, Department of Medical Microbiology, and Professor G. P. Royer, Department of Biochemistry for their helpful discussion.

I would also like to express my gratitude to my wife, Ok-rin, whose encouragement, sacrifice, and best companionship have enabled me to complete my graduate study.

Acknowledgment is made to the Ohio Agricultural Research and Development Center for financial support of the research.
VITA

June 6, 1944 . . . . Born - Seoul, Korea

1966 . . . . . . . . B.Sci. in Food Technology
Dongguk University, Seoul, Korea

1968-1970. . . . . Research Associate, Food Resources
Laboratory, Korea Institute of
Science and Technology, Seoul,
Korea

1970-1972. . . . . Graduate Research Associate,
Department of Nutritional
Sciences, University of
California, Berkeley,
California

1972 . . . . . . . . M.Sci. in Food Science
University of California,
Berkeley, California

1973-1976. . . . . Graduate Research Associate
Department of Food Science and
Nutrition, The Ohio State
University, Columbus, Ohio

PUBLICATIONS

Y. Park, E. M. Mikolajcik, and T. Kristoffersen, 1975
Coenzyme A Activity of Lactic Streptococci
J. Dairy Sci., 58:786

M. Plitman, Y. Park, R. Gomez, and A. J. Sinskey, 1973
Viability of Staphylococcus aureus in intermediate
Moisture Meats
J. Food Sci., 38:1004

Y. Park, M. M. Morris, and G. Mackinney, 1973
On Chlorophyll Breakdown in Senescent Leaves

M. M. Morris, Y. Park, and G. Mackinney, 1973
On the Photodecomposition of Chlorophyll in vitro
Production of Single-cell Protein on Petroleum Hydrocarbon, I. Isolation and Selection of Hydrocarbon Utilizing Microorganisms, Kor. J. Food Sci. Tech., 2:107


FIELDS OF STUDY

Major Field: Food Science

Studies in Food Science and Nutrition.
Professors W. J. Harper, T. Kristoffersen,
E. M. Mikolajcik, P. M. T. Hansen,
R. V. Josephson, and J. P. Kenyon

Studies in Biochemistry.
Professors R. A. Scott, G. P. Royer, and G. A. Barber

Studies in Microbiology.
Professors B. J. Kolodziej, G. D. Kagle, and D. A. Wolff

Studies in Immunology.
Professor M. C. Dodd

Professor R. M. Pfister

Studies in Physical Chemistry.
Professors Q. V. Winkle and M. H. Klapper
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>OBJECTIVES</td>
<td>4</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>5</td>
</tr>
<tr>
<td>The Organism</td>
<td>5</td>
</tr>
<tr>
<td>Habitat</td>
<td>6</td>
</tr>
<tr>
<td>Morphological characteristics</td>
<td>7</td>
</tr>
<tr>
<td>Biochemical characteristics</td>
<td>7</td>
</tr>
<tr>
<td>Factors affecting growth</td>
<td>8</td>
</tr>
<tr>
<td>Resistance to hostile environment</td>
<td>11</td>
</tr>
<tr>
<td>Enumeration of <em>Clostridium perfringens</em> in foods</td>
<td>12</td>
</tr>
<tr>
<td>The Toxins</td>
<td>15</td>
</tr>
<tr>
<td>Alpha toxin</td>
<td>16</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em> enterotoxin</td>
<td>22</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>27</td>
</tr>
<tr>
<td>Toxins and Antisera</td>
<td>27</td>
</tr>
<tr>
<td>Electroimmunodiffusion Technique</td>
<td>27</td>
</tr>
<tr>
<td>Radial Immunodiffusion Technique</td>
<td>28</td>
</tr>
<tr>
<td>Lecithovitellin Agar Test</td>
<td>28</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Hemolysin Indicator Plate Test.</td>
<td>29</td>
</tr>
<tr>
<td>Growth, Alpha Toxin Production and pH Development.</td>
<td>30</td>
</tr>
<tr>
<td>Polyacrylamide Gel Electrophoresis.</td>
<td>32</td>
</tr>
<tr>
<td>Heat Inactivation of Alpha Toxin.</td>
<td>34</td>
</tr>
<tr>
<td>Treatment of Data</td>
<td>35</td>
</tr>
<tr>
<td>RESULTS</td>
<td>36</td>
</tr>
<tr>
<td>Assay Procedures</td>
<td>36</td>
</tr>
<tr>
<td>Electroimmunodiffusion</td>
<td>36</td>
</tr>
<tr>
<td>Radial immunodiffusion</td>
<td>37</td>
</tr>
<tr>
<td>Lecithovitellin agar</td>
<td>37</td>
</tr>
<tr>
<td>Hemolysin indicator plate</td>
<td>40</td>
</tr>
<tr>
<td>Factors Affecting Hemolysin Indicator Test</td>
<td>48</td>
</tr>
<tr>
<td>Diluting agents</td>
<td>48</td>
</tr>
<tr>
<td>Type of agar</td>
<td>55</td>
</tr>
<tr>
<td>Minerals and sodium thioglycollate.</td>
<td>60</td>
</tr>
<tr>
<td>Chelating agents</td>
<td>69</td>
</tr>
<tr>
<td>Red blood cell(rbc) concentration</td>
<td>69</td>
</tr>
<tr>
<td>Incubation temperature and time</td>
<td>74</td>
</tr>
<tr>
<td>Commercial preparations</td>
<td>74</td>
</tr>
<tr>
<td>Bacterial origins</td>
<td>85</td>
</tr>
<tr>
<td>Growth, Alpha Toxin Production. and pH</td>
<td>85</td>
</tr>
<tr>
<td>Development of C. perfringens</td>
<td>88</td>
</tr>
<tr>
<td>Thioglycollate medium</td>
<td>88</td>
</tr>
<tr>
<td>Beef broth with ground beef and/or Promine-D</td>
<td>92</td>
</tr>
</tbody>
</table>
Population levels of *C. perfringens* and amount of alpha toxin produced. 99

*Selected Chemical and Physical Properties* 104

Polyacrylamide gel electrophoresis. 107

Heat inactivation. 118

DISCUSSION. 156

SUMMARY AND CONCLUSION. 169

REFERENCES. 174
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distribution of four major lethal toxins among the five types of <em>C. perfringens</em></td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Comparison of concentration of alpha toxin required to produce a minimum detectable reaction zone diameter</td>
<td>43</td>
</tr>
<tr>
<td>3</td>
<td>Composition of the Brewer Thioglycollate medium.</td>
<td>52</td>
</tr>
<tr>
<td>4</td>
<td>Mineral composition of the different types of agar.</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>Generation time, Gt; maximum yield of alpha toxin, Y; and time required for 90% reduction of alpha toxin, Dt, in Thioglycollate medium at different incubation temperatures</td>
<td>97</td>
</tr>
<tr>
<td>6</td>
<td>Comparison of generation time, Gt, and maximum alpha toxin yield, Y, in beef broth and 20% ground beef, and Thioglycollate medium at incubation temperature of 35°C</td>
<td>99</td>
</tr>
<tr>
<td>7</td>
<td>Selected characteristics of fractions isolated by polyacrylamide gel electrophoresis of two different commercial preparations of alpha toxin.</td>
<td>111</td>
</tr>
<tr>
<td>8</td>
<td>&quot;D&quot; values for alpha toxin in physiological saline and Thioglycollate medium.</td>
<td>121</td>
</tr>
<tr>
<td>9</td>
<td>The percent residual activity of alpha toxin when heated in physiological saline and Thioglycollate medium for 18 min.</td>
<td>124</td>
</tr>
<tr>
<td>10</td>
<td>The percent residual activity at 18 min of alpha toxin when heated in the individual components of Thioglycollate medium.</td>
<td>125</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>149</td>
<td></td>
</tr>
</tbody>
</table>

The percent residual activity at 18 min of alpha toxin heated in fractions of different molecular weights separated from Thioglycollate medium.

"D" values of alpha toxin when heated in the fractions obtained through molecular sieving of Thioglycollate medium.
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Quantification of alpha toxin from <em>C. perfringens</em> by the radial immunodiffusion technique</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>Quantification of the alpha toxin of <em>C. perfringens</em> by the lecithovitellin plate method</td>
<td>41</td>
</tr>
<tr>
<td>3</td>
<td>Quantification of alpha toxin of <em>C. perfringens</em> by the HI plate method where Thioglycollate medium was used as diluent for the alpha toxin</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>Sensitivity of the radial immunodiffusion (RID), lecithovitellin agar test(LV), and hemolysin indicator plate(HI) procedure for the quantification of the alpha toxin of <em>Clostridium perfringens</em></td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>HI test of <em>C. perfringens</em> alpha toxin</td>
<td>49</td>
</tr>
<tr>
<td>6</td>
<td>The effect of the diluting agents used for alpha toxin on the HI test</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>Effect of glucose(G), $K_2HPO_4$(K), sodium thioglycollate(T), beef extract(BE) and proteose peptone(PP) as diluting agents of alpha toxin on HI test</td>
<td>53</td>
</tr>
<tr>
<td>8</td>
<td>Effect of types of agar, Epi(Epiagar), Pan(Panagar), and Agar 3(Agar Agar No. 3), used in preparation of HI plates on HI test. (Saline used as diluent of alpha toxin.)</td>
<td>56</td>
</tr>
<tr>
<td>9</td>
<td>Effect of types of agar, Epi(Epiagar), Pan(Panagar), and Agar 3(Agar Agar No. 3), used in preparation of HI plates on HI test. (Thioglycollate medium used as diluent of alpha toxin)</td>
<td>58</td>
</tr>
<tr>
<td>10</td>
<td>Effect of 0.3 and 1.4% CaCl$_2$ incorporation in agar on HI test(Saline as diluent for alpha toxin)</td>
<td>61</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>11</td>
<td>Effect of 0.3 and 1.4% CaCl₂ incorporation in agar on HI test (Thioglycollate medium as diluent for alpha toxin)</td>
<td>63</td>
</tr>
<tr>
<td>12</td>
<td>Effect of 0.5% sodium thioglycollate (Th) or 0.3% MgCl₂ incorporation in agar on HI test (Saline as diluent for alpha toxin)</td>
<td>65</td>
</tr>
<tr>
<td>13</td>
<td>Effect of 0.5% sodium thioglycollate (Th) or 0.3% MgCl₂ incorporation in agar on HI test (Thioglycollate medium as diluent for alpha toxin)</td>
<td>67</td>
</tr>
<tr>
<td>14</td>
<td>Effect of red blood cell concentration on HI test (Saline as diluent of alpha toxin)</td>
<td>70</td>
</tr>
<tr>
<td>15</td>
<td>Effect of red blood cell concentration on HI test (Thioglycollate medium as diluent of alpha toxin)</td>
<td>72</td>
</tr>
<tr>
<td>16</td>
<td>Effect of length of incubation of HI plates at 21 C on HI test (Saline as diluent of alpha toxin)</td>
<td>75</td>
</tr>
<tr>
<td>17</td>
<td>Effect of length of incubation of HI plates at 35 C on HI test (Saline as diluent of alpha toxin)</td>
<td>77</td>
</tr>
<tr>
<td>18</td>
<td>Effect of length of incubation of HI plates at 21 C on HI test (Thioglycollate medium as diluent of alpha toxin)</td>
<td>79</td>
</tr>
<tr>
<td>19</td>
<td>Effect of length of incubation of HI plates at 35 C on HI test (Thioglycollate medium as diluent of alpha toxin)</td>
<td>81</td>
</tr>
<tr>
<td>20</td>
<td>Effect of different commercial sources of alpha toxin and different diluents used on the HI test</td>
<td>83</td>
</tr>
<tr>
<td>21</td>
<td>Comparison of lecithinase C from B. cereus and C. perfringens on HI test</td>
<td>86</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>22</td>
<td>Growth, pH, and alpha toxin production of <em>C. perfringens</em> in Thioglycollate medium at 25°C</td>
<td>89</td>
</tr>
<tr>
<td>23</td>
<td>Growth, pH, and alpha toxin production of <em>C. perfringens</em> in Thioglycollate medium at 35°C</td>
<td>91</td>
</tr>
<tr>
<td>24</td>
<td>Growth and alpha toxin production of <em>C. perfringens</em> in Thioglycollate medium at 45°C</td>
<td>93</td>
</tr>
<tr>
<td>25</td>
<td>Growth, pH, and alpha toxin production of <em>C. perfringens</em> in Thioglycollate medium at 50°C</td>
<td>95</td>
</tr>
<tr>
<td>26</td>
<td>Growth and toxin production of <em>C. perfringens</em> at 35°C in beef broth plus 20% ground beef or beef broth plus 14% ground beef and 6% Promine-D</td>
<td>100</td>
</tr>
<tr>
<td>27</td>
<td>Relationship between viable counts of <em>C. perfringens</em> and alpha toxin production in Thioglycollate medium at different incubation temperatures</td>
<td>102</td>
</tr>
<tr>
<td>28</td>
<td>Relationship between viable counts of <em>C. perfringens</em> and alpha toxin production in Thioglycollate medium and beef broth with 20% ground beef</td>
<td>105</td>
</tr>
<tr>
<td>29</td>
<td>Polyacrylamide disc gel electrophoresis of alpha toxin of <em>C. perfringens</em></td>
<td>108</td>
</tr>
<tr>
<td>30</td>
<td>Densitometer scans obtained from the electrophoretogram of Wellcome alpha toxin</td>
<td>109</td>
</tr>
<tr>
<td>31</td>
<td>Densitometer scans obtained from the electrophoretogram of Calbiochem alpha toxin</td>
<td>110</td>
</tr>
<tr>
<td>32</td>
<td>Hemolytic test of non-dialyzed and dialyzed fractions obtained upon polyacrylamide gel electrophoresis of Wellcome's alpha toxin preparation</td>
<td>113</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Hemolytic test of non-dialyzed and dialyzed fractions obtained upon polyacrylamide gel electrophoresis of Calbiochem's alpha toxin preparation.</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Hemolytic test of alpha toxin of <em>C. perfringens</em> on the horse rbc-HI plate.</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>Hemolytic test of alpha toxin of <em>C. perfringens</em> on the horse rbc-HI plate.</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>Percentage of alpha toxin remaining after heating at temperature of 55-95°C in physiological saline and in Thioglycollate medium.</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>Thermal inactivation time curve of alpha toxin in physiological saline and Thioglycollate medium.</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Percentage of alpha toxin remaining after heating at temperature of 55-95°C in 0.5% glucose.</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>Percentage of alpha toxin remaining after heating at temperature of 55-95°C in 0.05% sodium thioglycollate.</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Percentage of alpha toxin remaining after heating at temperature of 55-95°C in 0.6% beef extract.</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>Percentage of alpha toxin remaining after heating at temperature of 55-95°C in 0.2% K$_2$HPO$_4$.</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>Percentage of alpha toxin remaining after heating at temperature of 55-95°C in 1% proteose peptone.</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>Hemolytic test of alpha toxin after heating time of 0, 3, 6, 9, 12, 15, 18 min at 95°C.</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>Percentage of alpha toxin remaining after heating at temperature of 55-95°C in the fraction of MW&lt;1,000 obtained from Thioglycollate medium.</td>
<td>138</td>
</tr>
<tr>
<td>45</td>
<td>Percentage of alpha toxin remaining after heating at temperature of 55-95°C in the fraction of 1,000&lt;MW&lt;10,000 obtained from Thioglycollate medium.</td>
<td>140</td>
</tr>
<tr>
<td>46</td>
<td>Percentage of alpha toxin remaining after heating at temperature of 55-95°C in the fraction of 10,000&lt;MW&lt;30,000 obtained from Thioglycollate medium.</td>
<td>142</td>
</tr>
<tr>
<td>47</td>
<td>Percentage of alpha toxin remaining after heating at temperature of 55-95°C in the fraction of MW&gt;30,000 obtained from Thioglycollate medium.</td>
<td>144</td>
</tr>
<tr>
<td>48</td>
<td>Thermal inactivation time curve of alpha toxin in the fractions of different molecular weights separated from Thioglycollate medium.</td>
<td>147</td>
</tr>
<tr>
<td>49</td>
<td>Percent residual activity of alpha toxin of culture supernatant of C. perfringens after heating at temperature of 55-95°C.</td>
<td>151</td>
</tr>
<tr>
<td>50</td>
<td>Percentage of alpha toxin remaining after heating at temperature of 55-95°C in 0.01 M CaCl₂.</td>
<td>153</td>
</tr>
</tbody>
</table>
INTRODUCTION

*Clostridium perfringens* is the third most common reported cause of food poisoning in man. In 1974, it accounted for 9.4% of the confirmed cases of foodborne disease outbreaks. Many cases go unreported. The symptoms of food poisoning occur 6-24 hrs after the ingestion of the contaminated food, usually within 8-12 hrs. Symptoms consist of abdominal cramps and diarrhea. Nausea and vomiting are rare. Mishandled cooked meat and meat products in particular are most often involved. In contrast to staphylococcal food poisoning and botulism, *C. perfringens* food poisoning requires ingestion of large numbers of vegetative cells rather than of a preformed toxin. About $10^9$ *C. perfringens* cells in the vegetative state are sufficient to produce the food poisoning syndrome in man. Therefore, cell numbers are important in the investigation of foodborne outbreaks.

However, there are inherent problems in the enumeration of *C. perfringens*. The organism is an anaerobe. Therefore, special equipment and techniques are necessary to achieve anaerobiosis. Enumeration of *C. perfringens* in suspected food samples requires various selective agents e.g. antibiotics, sulfite and iron compounds. Some of these agents also inhibit the *C. perfringens* organisms or in other
cases permit the outgrowth of unrelated organisms. Thus, the ideal medium for the isolation and enumeration of *C. perfringens* from food systems has not as yet been devised.

*Clostridium perfringens* is known to be susceptible to adverse conditions. It will die off rapidly in refrigerated or frozen foods. Consequently, low numbers of organisms in foods may elude detection. Under favorable conditions, even these low numbers could subsequently outgrow to potentially hazardous levels.

Ideally, it would be desirable if we could exploit some unique metabolite of the *C. perfringens* organism which could be detected readily, remain stable under various food processing and storage conditions, and whose presence would accurately reflect the population level which the organisms had attained in the food. The ultimate goal would be to develop a rapid and simple chemical test analogous to the thermal stable nuclease procedure used for the detection in foods of viable *Staphylococcus aureus* or of their prior growth.

The metabolite, alpha toxin (E.C.3.1.4.3. Phosphoglyceride diglyceride-hydrolase; Lecithinase C, or Phospholipase C) of *C. perfringens* meets many of the above requirements. Although, it is not involved *per se* in food poisoning by *C. perfringens*, some workers have suggested the possibility that alpha toxin could be used as an indirect measure of *C. perfringens* organisms.
This study, therefore, proposes to increase our knowledge of the alpha toxin of *C. perfringens* with respect to rate of production by the organism, quantification procedures, and physical and chemical factors affecting its activity.
OBJECTIVES

The major objectives of the study were:

1. To ascertain whether or not alpha toxin can be used for the estimation of population levels of *C. perfringens*.

2. To evaluate various quantification procedures for alpha toxin and factors affecting their sensitivity.

3. To correlate the growth of *C. perfringens* and alpha toxin production under laboratory conditions, and

4. To study selected chemical and physical properties of alpha toxin.
REVIEW OF LITERATURE

The review of literature will deal with the organism
Clostridium perfringens and its two major toxins.

The Organism

Clostridium perfringens was described, as early as
1891, by Achalme (Smith and Holdeman, 1968). In 1892, it was
named Bacillus aerogenes capsulatus by Welch and Nuttal.
Andrews (1899) reported a food-borne illness incident attribu-
ted to this organism. In the United States, the involvement
of C. perfringens in human food poisoning was clearly esta-
blished in 1945 by McClung. The term "perfringens" is deriv-
ed from the Latin and means "breaking through". In medical
literature, it is often referred to as Clostridium welchii
or "the gas bacillus".

Numerous synonyms for the organism have been used.
These include: Bacillus aerogenes capsulatus, Bacillus ent-
eritidis sporogenes, Bacillus cadaveris butyricus, Bacillus
perfringens, Bacillus phlegmonis emphysematosa, Bacillus
saccharobutyricus immobilis, Bacillus vaginae emphysematose,
Bacillus welchii, Granulo-bacillus butyricus, and Clostri-
dium welchii.
Clostridium welchii is preferred by Europeans and Clostridium perfringens by Americans.

**Habitat:** Smith and Holdeman (1968) consider C. perfringens more ubiquitous than any other pathogen. It is found in water, milk, soil, spices, fish, vegetables, market meats, dehydrated soups, clothing, and skin. However, its principal habitats are soil and the intestinal contents of man and animals. The species is divided into 5 types, A to E, on the basis of major lethal toxins produced. Only Type A is found in both soil and intestines of man and animals. The other types seem to be obligate parasites mostly of animals (Smith and Holdeman, 1968). If intestinal parasite types (i.e. Type B, C, D or E) are added to the soil, they will die out shortly indicating that they are unable to compete with Type A which is endogenous to soil.

Type B, C and D have been found in the intestines of man and animals. In addition, Type E has been found in animals only. Apparently, these potentially toxigenic organisms can survive for long periods in the intestinal tracts without causing appreciable damage to the host. Since demonstrable amounts of antisera to these toxins are sometimes detected in animals without any previous history of immunization, they may serve to immunize their hosts naturally against toxins they produce.
Morphological characteristics: Clostridium perfringens is a non-motile, encapsulated, short and thick bacillus with blunt ends, measuring 2 to 4 \( \mu \text{m} \) in length and 0.8 to 1.5 \( \mu \text{m} \) in width. Cells occur singly, in pairs and occasionally as short chains. Filamentous cells are sometimes produced. Young cultures stain gram-positive, while old cultures may appear as gram-negative. They are sporeformers, although in vitro, sporulation is poor. The spores are mostly oval and subterminal (Prevot, 1966; Bryan, 1969; Smith and Holdeman, 1968; Buchanan and Gibbons, 1974).

Colonies on blood agar are convex, semi-opaque, glossy with entire margins when incubated overnight (Smith and Holdeman, 1968). Colonies incubated longer will have a raised center with flattened periphery.

All types of C. perfringens form colonies of very similar appearance. Those of Types B and D may be somewhat smaller than those of Type A. However, variation exists from strain to strain within a single type and no dependable generalization can be made.

Biochemical characteristics: Most strains of C. perfringens ferment glucose, maltose and dextrin. Starch is hydrolyzed but hydrolysis may be incomplete. \( \text{H}_2\text{S} \) is formed in most media; indol is not produced; and nitrate reduction is
variable depending upon the basal medium. Reduction of nitrate is dependent upon the presence of a hydrogen donor. Most strains can liquefy gelatin, but not casein or albumin. The enzyme involved appears to be neither kappa or lambda toxin (Mansson and Smith, 1962).

Factors affecting growth: Clostridium perfringens requires at least 13 amino acids for growth and multiplication. In addition, 5 to 6 growth factors are also necessary (Boyd et al., 1948; Fuchs and Bonde, 1957; Sebold and Costilow, 1975). These nutritional needs may play a role in toxin production. Foods associated with outbreaks caused by C. perfringens are usually high in protein, e.g. meat or meat dishes.

Clostridium perfringens is not a strict anaerobe since it can tolerate some oxygen. The optimum redox potential ($E_h$) of the culture is approximately -200 mv (Reed and Orr, 1943). However, Smith and Holdeman (1968) reported that it may grow in media with $E_h$ up to +230 mv at pH 6.0. The specific redox potential supporting growth of the organism may vary with the strain, inoculum size, pH, and metabolic state. For example, Mead (1969) found the limiting $E_h$ to be +194 to +238 mv when 0.5% NaCl was present and pH of media was 7.0-7.2. However, when the salt concentration was increased to 5%, the $E_h$ had to be lowered to +66 mv for initiation of
growth. An $E_h$ of +200 mv at pH 7.0 was also reported by Tabatabai and Walker (1970) as supporting growth.

The optimum temperature for growth of *C. perfringens* has been reported as 43-46 C (Boyd, *et al*., 1948; Collee, *et al*., 1961; Smith and Holdeman, 1968). It has been noted that while most rapid growth occurs between 43-46 C, 37 C will produce higher counts (Collee, *et al*., 1961; Strong, *et al*., 1970). The temperature range over which it grows readily is 20 to 50 C. Although the temperature optimum for growth is described in the 43 to 46 C range; this is not necessarily the optimum temperature for production of enzymes or toxins by *C. perfringens*. For example, the temperature optimum for the production of phospholipase C (alpha toxin) was found to vary from 30 to 46 C depending on the strains, while that for epsilon toxin production was 30 to 33 C (Nakamura, *et al*., 1969). The term "the Phoenix phenomenon" was coined by Collee *et al*.(1961) to describe a curious fact observed with the growth at 50 C. At this high temperature, most of the inoculum will die in the first few hours. The survivors, however, will shortly begin multiplying at almost the maximum growth rate.

Barnes *et al*.(1948) found growth in beef at 20 C at a pH of 5.7-5.8 and rapid growth at pH 7.2. After a long lag period, growth began in beef cubes with gravy at 18.3 C (Hall
and Angellotti, 1956). The optimal temperature for sporulation was found to be 37°C (Kim, et al., 1967). The optimum temperature for germination was reported to be 30°C (Ahmed and Walker, 1971).

The pH of a medium can also affect the growth of C. perfringens. Most strains grew well in a pH range of 5.5 to 8.0, with no growth occurring below pH 5.0 or above pH 8.0 (Fuchs and Bonde, 1957; Smith and Holdeman, 1968). Little, if any, variation in growth was observed in a pH range of 6.0 to 7.5 (Smith, 1963). Prevot (1966) listed an optimum pH for growth as 6.8, whereas Barnes et al. (1963) obtained optimum growth at 7.2.

The optimum pH for germination is 6.0 (Ahmed and Walker, 1971) and that for sporulation 6.6–6.8 (Torrey, et al., 1930). The pH optimum for production of (a) phospholipase C is slightly below 7.0 (Pivnick, et al., 1963); (b) beta toxin and delta toxin is 7.5; and (c) epsilon toxin and theta toxin is 7.0 to 7.2 (Pivnick, et al., 1965).

The effect of water activity (Aw) or solute concentration on growth of C. perfringens has been studied by several workers, including Gough and Alford (1965), Kang, et al. (1969), Mead (1969), and Strong, et al. (1970). Kang, et al. (1969) found the lowest water activity for growth to be between 0.97 and 0.95 with sucrose or sodium chloride, or 0.93 with glycerol.
Strong, et al. (1970) found 0.96 as the limiting water activity when using glucose as a solute.

Generation time varies depending on how optimal growth requirements are rendered to the organism. Generation time in meat stock soup has been reported to be 24-32 min. (Smith, 1963). Bryan, et al. (1971) found it as short as 8.5 min. in a favorable medium.

Resistance to hostile environment: Barnes et al. (1963) studied the effects of low temperatures on the survival of C. perfringens in frozen meat. It was found that spores were resistant to freezing at -5 C or -20 C. Vegetative cells were more sensitive to freezing temperatures. A slow destruction of vegetative cells occurred at 1, 10, and 15 C, but spores were stable at these temperatures. In laboratory media, Canada et al. (1964) recovered 16-58% of spores subjected to -17.7 C and 7.1 C for 48 hrs.

After similar treatment, only small numbers of the vegetative cells were recovered. Strong and Canada (1964) reported that in frozen chicken gravy 4.29% and 3.69% of viable cells survived for 90 and 180 days, respectively. When spores were added to the gravy, maximum survival was 37.9% at 90 days and 10.9% at 180 days. Other investigators have observed decreases in viability during refrigerated storage.
The survival of *C. perfringens* during cooking has been studied. Woodburn and Kim (1966) found that during cooking of the stuffed turkeys in an oven at 94°C, vegetative cell count declined steadily while the spore counts remained constant or increased slightly. Strong and Ripp (1967) noted that the organism could survive better when the internal temperature reached was 74°C than 85°C. Counts remained essentially the same when the food was held at 68°C for 6 hrs.

In a thioglycollate broth, 6% NaCl was needed to inhibit *C. perfringens* (Gough and Alford, 1965). Mead (1969) found four strains that were able to grow in 6% NaCl within 14 days at 37°C. One strain (NCTC 8237) grew in 7% NaCl, but only from an initial high inoculum (10^5-10^6 organisms/ml).

**Enumeration of *C. perfringens* in foods**: In the investigation of cases of food poisoning by *C. perfringens*, it is required to examine both suspected foods and feces of patients. *Clostridium perfringens* may be enumerated by the most probable number (MPN) technique or by colony counts on solid agar media. The MPN technique has several disadvantages: it is laborious, has a relatively low precision, and is susceptible to contamination from the laboratory environment. Therefore, the method is not commonly used.

Several selective media have been devised for isolating
C. perfringens from samples contaminated with other bacteria. Most of the selective media for enumeration of C. perfringens contain iron salts and sulfite. The latter is reduced by clostridia to sulfide. The resulting precipitate of iron sulfide produces black colonies. These media also incorporate antibiotics to inhibit the growth of other organisms which may produce black colonies and create a false count (Spencer, 1969). Counts have been carried out in Petri dishes, deep agar tubes, and plastic pouches (Bladel and Greenberg, 1965). Petri dishes are most commonly used, since only a few colonies can be picked from deep agar tubes and the preparation and handling of the plastic pouches is tedious.

Angelotti et al. (1962) devised a medium which was a modification of Mossel's medium. Sodium sulfadiazine was added to the already present Polymyxin sulfate and sodium sulfite. This addition inhibited the growth of Proteus sp., Pseudomonas sp. and coliforms. It was named Sulfite-Polymyxin-Sulfadiazine (SPS) agar. Some discrepancies in counts were reported when commercially prepared medium was compared with laboratory prepared medium (Hauschild, et al., 1967a).

Marshall et al. (1965) substituted Neomycin sulfate for the sulfadiazine and increased the concentration of the polymyxin. This medium was named Tryptone-Sulfite-Neomycin (TSN) agar. An incubation temperature of 46 C was recommended to
limit the growth of other sulfite-reducing clostridia such as Clostridium bifermentans.

Shahidi and Ferguson (1971) used Kanamycin and Polymyxin B as the principal selective agents in their Shahidi-Ferguson-Perfringens (SFP) agar. In addition to ingredients permitting demonstration of $H_2S$ production, this medium contains egg yolk to detect lecithinase production by C. perfringens. On this agar, therefore, the black colonies would be surrounded by an opaque zone. Harmon et al. (1971b) modified this medium by substituting D-Cycloserine for Kanamycin and Polymyxin. This medium is known as Tryptose-Sulfite-Cycloserine (TSC) agar.

All of these media have been compared for their ability to selectively quantitate C. perfringens. TSN is the most selective; however, it is also inhibitory to several strains of C. perfringens (Harmon et al., 1971a). SFP agar is the least inhibitory to C. perfringens but it is not very selective against a large number of facultative anaerobes (Harmon et al., 1971a; Hauschild and Hilsheimer, 1974). Therefore, if C. perfringens is to be isolated quantitatively from highly contaminated food samples, probably the most selective of the media, TSN agar, would be most satisfactory, even though the counts might be somewhat low. Highest counts would probably be achieved on SFP agar, but its use might encounter difficulties when C. perfringens was greatly out-
numbered by other organisms, because this medium is the least selective.

All isolates should be confirmed morphologically and biochemically. Angelotti et al. (1962) suggested a confirmatory test, i.e. growing stab cultures from an adequate number of black colonies in nitrate-motility agar. *C. perfringens* is nonmotile and forms a line of growth along the stab. It reduces nitrates to nitrites. Reduction of nitrate to nitrite is tested by adding sulfanilic acid and alpha-naphthylamine to the tube. A bright red color in the upper portion of the stab indicates the presence of nitrite.

**The Toxins**

Smith and Holdeman (1968) listed 12 separate toxic components in culture filtrates of *C. perfringens* that have been identified by other researchers. The identified toxins are: alpha, beta, gamma, delta, epsilon, eta, theta, iota, kappa, lambda, mu, and nu. All of these toxins are not necessarily always present in the same strain. Different strains will have varying proportions of toxins. All of these toxins appear to be proteins and some are enzymes. They are inactivated by heat and formaldehyde; are antigenic and combine with their antibodies in definite proportions (Smith and Holdeman, 1968). Strains of *C. perfringens* are divided into five
types on the basis of the production of four major lethal toxins: alpha, beta, epsilon, and iota as shown in Table 1.

Only Types A and C have been implicated in food-poisoning outbreaks (Sterne and Warrack, 1964). Type A strains also cause gas gangrene. Type C has caused outbreaks of necrotic enteritis in Germany (Oakley, 1949) and in New Guinea (Murrell, et al., 1966).

Types B, C, and D have caused enterotoxemia in animals including sheep, calves, and goats (Smith and Holdeman, 1968).

Whether or not Type E is responsible for disease in either animals or man has not been demonstrated conclusively (Smith and Holdeman, 1968).

Table 1. Distribution of four major lethal toxins among the five types of C. perfringens (a)

<table>
<thead>
<tr>
<th>Type</th>
<th>Alpha</th>
<th>Beta</th>
<th>Epsilon</th>
<th>Iota</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

(a) Smith and Holdeman (1968)

**Alpha toxin:** Alpha toxin (E.C.3.1.4.3., Phosphoglyceride
diglyceride-hydrolase, Lecithinase C, or Phospholipase C) is a toxigenic protein produced by all types of *C. perfringens*. It is hemolytic, necrotizing, and lethal; splitting lecithin to phosphoryl choline and a diglyceride.

The specificity of the phospholipase activity of alpha toxin to various phosphatides has been the subject of considerable discussion. While Zamecnik *et al.* (1947) indicated that sphingomyelin, phosphatidyl ethanolamine, phosphatidyl serine and lysolecithin were not attacked, Macfarlane (1948) and Matsumoto (1961) later reported enzyme activity that decomposes phosphatidyl ethanolamine as well as sphingomyelin in a culture filtrate. Matsumoto (1961) suggested the possibility that the alpha toxin preparation of *C. perfringens* may contain different enzymes that attack lecithin, sphingomyelin, and cephalin.

It is possible to measure the activity of alpha toxin in a number of ways. The ability of alpha toxin (a) to cause an increase in the turbidity of an egg yolk suspension, (b) to lyse red blood cells, (c) to release acid-soluble phosphorus from lecithin, and (d) to liberate acid groups consequent to the breakdown of lecithin maybe utilized.

*C. perfringens* alpha toxin is a so-called "hot-cold" hemolysin, i.e. if a low concentration of toxin is incubated with erythrocytes at 37°C no hemolysis takes place, but when
the suspension is cooled the erythrocytes hemolyse. There is considerable variation from one species to another in red blood cell susceptibility to lysis by alpha toxin. The red blood cells of cattle and mice seem most susceptible. Those of rabbits, sheep, and men are moderately susceptible, whereas, those of horses and goats are comparatively resistant. Apparently, the susceptibility or resistance of red blood cells to lysis by alpha toxin is determined by either (a) the type of phospholipid, i.e. lecithin or sphingomyelin, or (b) its location in the cell membrane.

When it is injected intravenously in susceptible animals like rabbits or rats, alpha toxin causes marked intravascular hemolysis (Macfarlane and MacLennan, 1945). Hemolysis is probably caused by gross alteration of cell structure and function when phospholipids are split into phosphoryl choline and diglyceride.

The discovery of the enzymatic nature of alpha toxin led to a detailed study of the properties of this enzyme, including the determination of specific inhibitors and activators of its activity. It has been noted that calcium is required for lecithinase activity (Macfarlane and Knight, 1941, and Zamecnik, et al., 1947). According to the data of Zamecnik et al. (1947), increase in the concentration of CaCl₂ up to 0.1 M produced activation, but a further increase produced
inactivation of the enzyme. Similar results were observed with an increase in magnesium concentration of 0.001 M. The activity of alpha toxin is inhibited by chelating agents, such as phosphate, citrate, or EDTA, that tightly bind calcium rendering it unavailable to release a positive charge on the substrate.

The enzymatic activity of alpha toxin seems to depend on the electrokinetic characteristics of the substrate and the enzyme. The substrate needs to be positively charged and the enzyme negatively charged. The activation of lecithin breakdown by divalent cations such as calcium and magnesium seems to depend on their producing a positive charge on the surface of the lecithin micelles (Bangham and Dawson, 1961).

Alpha toxin is irreversibly inhibited by certain reducing agents such as cysteine, thioglycollic acid, glutathione, hydrogen sulfide and sodium bisulfite (Smith, 1955). The mechanism of this action may be associated with one or more disulfide bonds whose integrity is essential to enzymatic activity. The action of the reducing agents may be to reduce the disulfide linkages to sulfhydryl groups (Smith, 1955). The resistance of alpha toxin to thiol poisons such as p-chloromercuribenzoate and monoiodoacetate also supports the fact that the lecithinase is not a sulfhydryl enzyme dependent
for its activity on sulfhydryl groups (Ispolatovskaya, 1971).

Although the alpha toxin produced by different Type A strains is quite similar, it apparently is not identical. Preparations from different strains show varying ratios of biological activity to enzymatic activity ($LD_{50}$/lecithinase activity). In four strains examined, these ratios varied from 1.56 to 4.5 (Dolby and MacFarlane, 1956). It seems likely that the differences are due to small dissimilarities in the configuration of the alpha toxin or possibly to the production of several slightly different lecithinases in varying proportions by the different strains.

Alpha toxin is produced by strains of all types of C. perfringens, but seems to be produced in greatest amount by strains of Type A. The composition of the nutrient medium strongly influences the toxin production of this organism. Growth is a prerequisite for toxin synthesis, but some factors with little effect on growth have a profound effect on toxigenesis. For example, several authors (Jayko and Lichstein, 1959; Tsukamoto, et al., 1963; Hauschild, 1965b) have demonstrated that high yields of toxins in cultures of C. perfringens depend on an adequate supply of peptides, although growth may be the same in media containing either acid hydrolysates or enzyme hydrolysates of protein. While the presence of arginine was essential for C. perfringens to produce alpha toxin
(Murata, et al., 1965), cystine was reported to inhibit alpha toxin production (Gooder and Gehring, 1954). The addition of a combination of vitamins, zinc, and magnesium to the medium stimulated toxin production (Murata, et al., 1965). Nakamura, et al. (1969) reported that the addition of lecithin to the culture medium enhanced toxin production. The effect of pH on toxigenesis of C. perfringens was studied by Pivnick, et al. (1965). They found that optimum pH was 7.0 to 7.2 for alpha toxin. The literature is in disagreement regarding the composition of the nutrient medium that should be used. Resolution of this question is difficult because the widely used meat and casein hydrolyzates are not standardized. Furthermore, growth and toxin production of C. perfringens was not determined by the same procedures.

The molecular weight of alpha toxin has been reported as 30,000 (Mollby and Wadstrom, 1973), 51,200 (Shemanova, et al., 1968), 90,000 (Casu, et al., 1971), 100,000 (Ikezawa, et al., 1964) or 106,000 (Meduski and Volkova, 1957). The great discrepancies in the value of the molecular weight of alpha toxin reported in the literature might suggest that different molecular forms exist, or that different strains produce different enzymes.

Smith and Gardner (1950) found that there was greater heat inactivation of alpha toxin at 65 °C than at 100 °C. This observation, however, could not be completely confirmed
by Nakamura, et al. (1969). Harmon and Kautter (1970) reported that the activity of alpha toxin remained unchanged in some food samples stored for 3 months at -20 C.

A method has been proposed, in which the quantity of alpha toxin detected can be utilized to estimate the extent of previous growth of the organism by using the relationship between viable count and the amount of alpha toxin produced (Harmon and Kautter, 1970; Harmon and Kautter, 1974).

A similar concept is being promoted for Staphylococcus aureus by Tatini, et al. (1976) among others. They suggest that heat-stable staphyloccocal deoxyribonuclease (DNase), which is produced by a majority (>98%) of enterotoxigenic S. aureus, could serve as an indicator of S. aureus growth.

C. perfringens enterotoxin. Although C. perfringens has been identified as a responsible agent of food poisoning for a quite long time, the sequence of events which lead to the illness has not been thoroughly understood. It has been shown that while a few cells of C. perfringens suffice to induce gas gangrene, the number of cells required to induce food poisoning is of the order of $10^8$-$10^9$ (Dische and Elek, 1957; Hauschild and Hatcher, 1967; Hobbs, 1965).

According to Hobbs (1965) and Sterne and Warrack (1964), strains of Type A C. perfringens can be divided into two groups: (a) the classical strains causing gas gangrene in man and animals and producing heat-susceptible spores, and
(b) the food-poisoning strains producing heat-resistant spores. For some years it was generally thought that only heat-resistant strains are associated with food poisoning outbreaks (Hobbs, 1965). However, several workers have shown that strains which produced heat-sensitive spores could also produce the same symptoms that had originally been attributed to heat-resistant strains (McKillop, 1959; Hall, et al., 1963; Hauschild and Thatcher, 1967). It was concluded that the division into "classical" and "food-poisoning" strains was no longer valid.

Nygren (1962) has pointed out that the enteropathogenic factor responsible for C. perfringens food poisoning was alpha toxin (lecithinase C). However, this has been disproved conclusively by Weiss, et al. (1966), Duncan, et al. (1968) and Hauschild, et al. (1968).

Suitable animals researched, for use as test subjects in which the events that lead to food poisoning in humans can be duplicated, have included monkeys (Duncan and Strong, 1971; Hauschild, et al., 1971c), lambs and sheep (Hauschild, et al., 1967; Niilo, et al., 1971), and rabbits (Duncan and Strong, 1969a).

In lambs and rabbits, diarrhea has been produced experimentally (Hauschild, et al., 1967c; Duncan and Strong, 1969a; Niilo, et al., 1971). It was found that vegetative cells suspended in fresh media were required to elicit a response. Cultural supernatants from the vegetative cells
had no effect (Hauschild, et al., 1967c; Duncan and Strong, 1969a), whereas sporulating cells and their extracts could produce the symptoms (Duncan and Strong, 1969b; Hauschild, et al., 1970a).

Besides using the animals in their natural state to imitate the food-poisoning syndrome, a localized response could also be produced by the development of the ligated ileal loop technique in both rabbits and lambs (Duncan, et al., 1968; Hauschild, et al., 1968; Duncan and Strong, 1969b; Hauschild, et al., 1971a; Strong, et al., 1971). If a preparation containing the enteropathogenic factor is introduced into the ligated loop, the loop will distend and give a sausage-like appearance. As in the production of diarrhea, a loop distention was obtained only by the combination of vegetative cells and fresh medium. Cells without nutrient medium or medium without cells had no effect.

Since vegetative cells alone could not produce a positive response this indicated that the causative factor of the enteritis was not a component of the vegetative cells. However, the agent was present in cell extracts and supernatant fluids of sporulated cultures (Duncan and Strong, 1969b; Hauschild, et al., 1970b). The factor has been found to locate exclusively in the cytoplasm of young sporulated cultures (Hauschild, et al., 1970b). Toxin production has been shown to be sporulation specific by Duncan,
et al. (1972).

Intradermal injection of the toxin into guinea pigs and rabbits caused erythema around the injection site within one to two hours (Hauschild, 1970b). The reaction was characterized by the absence of necrosis and was distinctly different from the skin reactions of all the other known toxins of C. perfringens. Niilo (1971) found that increased capillary permeability occurred around the injection site before the erythematous response was evident. Through the use of immunodiffusion it has been demonstrated that the enteropathogenic and erythematous factor produced both in vivo and in vitro are one and the same substance. This substance has been named Clostridium perfringens enterotoxin (Hauschild, et al., 1971b).

Little is known about the mode of action of the enterotoxin in vivo. Niilo (1971) suggested that increased capillary permeability, increased vasodilation and increased intestinal motility as the sequence of changes that would result in diarrhea in the normal intestine or fluid accumulation in the ligated ileal loop.

The enterotoxin could be completely neutralized with antiserum prepared in rabbits or sheep against crude extracts of sporulated C. perfringens cells (Hauschild, et al., 1970a). Although the enterotoxin was completely neutralized in vitro, circulating antibodies produced from injections of the ente-
rotoxin had no protective effect against enterotoxin produced in the intestine. Niilo, et al. (1971) was not able to establish protective immunity in sheep. The immunized animals were still susceptible to infection. Also no measurable circulation of antibodies could be detected in animals that were repeatedly challenged over a period of time.

The *C. perfringens* enterotoxin was purified and characterized by several researchers (Hauschild and Hilsheimer, 1971; Stark and Duncan, 1972). However, it is beyond the scope of this review to deal with the chemical and physical properties of the enterotoxin in detail.

In summary, Hauschild (1971) suggested the following sequence of events leading to *C. perfringens* food poisoning; (a) ingestion of food contaminated with large numbers of vegetative *C. perfringens*; (b) multiplication and sporulation of *C. perfringens* in the small intestine; (c) production of enterotoxin associated with sporulation and release of the toxin by cell lysis; (d) increased capillary permeability and vasodilation in the intestine, and possibly, increased intestinal motility; and (e) excess fluid accumulation in the intestinal lumen resulting in diarrhea.
MATERIALS AND METHODS

Toxins and Antisera

Alpha toxin of *C. perfringens* was obtained from Wellcome Reagents Limited, Beckenham, England, and from Calbiochem, San Diego, California. Unless otherwise stated, alpha toxin obtained from Wellcome Reagents Limited was used throughout this study.

Phospholipase C of *Bacillus cereus* was obtained from Calbiochem, San Diego, California.

Anti-alpha toxin was obtained from Wellcome Reagents Limited, Beckenham, England.

Electroimmunodiffusion Technique

For EID, basically the method of Laurell (1966) was used. The cellulose acetate plates (78 x 98 mm, Helena Laboratory, Beaumont, Texas) were soaked for 10 min. in appropriate anti-alpha toxin diluted in 0.0125 M phosphate buffer, pH 7.4. Samples of 0.4 μl were spotted in duplicate on the plate with a one μl Hamilton microsyringe. To remove unreacted proteins, the plate after electrophoresis was washed for one hr with 0.0125 M phosphate buffer, pH 7.4, containing 0.2 M sodium chloride.

After rinsing with distilled water, the plate was stained
with Nigrosin (0.125% in 6% acetic acid). It was then rinsed with distilled water to remove excess dye, washed with 6% acetic acid, and rinsed again with distilled water.

**Radial Immunodiffusion Technique**

One tablet of Panagar (Oxoid, I.D. agar, BR 27) was added to 50 ml distilled water. After soaking for 15 min., it was placed in boiling water bath to dissolve the agar with occasional shaking. After it was completely dissolved, it was cooled to 45-47°C. The antiserum of alpha toxin was added at the concentration of 1% and mixed well. The agar-antiserum mixture (10 ml) was poured in a plastic mold (10 x 5 cm) and was allowed to solidify. Just prior to use, test wells (3 mm) were cut into the agar with a sterile thin-walled metal die.

Five microliters of each sample were applied in the test wells of antiserum-agar plates. The plates were incubated for 48 hrs in a humidified cool (7°C) chamber. The diameter of precipitin ring developed was measured in mm, using a microcalipher. Weak precipitin rings of insufficient contrast were stained with 0.1% thiazine red in 1% acetic acid.

**Lecithovitellin Agar Test**

Lecithovitellin solution was prepared by mixing egg yolk in physiological saline to obtain 20% suspension in volume,
centrifuging at 15,000 RCF for 15 min., and sterilizing the supernatant by Seitz filtration. Agar(1.7%) was prepared by dissolving Agar Agar No. 3(Oxoid) in physiological saline. The agar(90 ml) was cooled to 45-47°C and 10 ml of the lecithovitellin solution was added and mixed well. Ten milliliters of the lecithovitellin-agar solution was dispensed in Petri dishes(100 x 15 mm) and allowed to air cool. The test wells were prepared in the same way as described in the Mancini radial immunodiffusion technique. Samples(5 μl) were then applied in the test wells of the lecithovitellin-agar plates. The plates were incubated at 37°C for 24 hrs. The diameter of reaction zones was then measured as described above.

**Hemolysin Indicator(HI) Plate Test**

(a) Saline agar base: Seventeen grams of Agar Agar No.3 (Oxoid) and 8.5 g NaCl were added to 1 l distilled water. The pH was adjusted to 7.0, and the agar was dissolved by heating, followed by autoclaving for 15 min. at 121°C.

(b) Washed red blood cells: Packed human red blood cells (rbc) were washed 3 times by mixing with 4 volumes of sterile physiological (0.85%) saline and centrifuging for 15 min. at 3,000 RCF. Sterile precautions were practiced. The rbc were obtained from the American Red Cross Blood Bank, Columbus, Ohio.
(c) **Preparation of HI plates.** Twelve milliliters of washed rbc were added to 100 ml of saline agar base at 55 c, mixed thoroughly, and dispensed 10 ml per each 100 x 15 mm petri dish. The rbc concentration of the plates was approximately $16.8 \times 10^6$ rbc/ml. The plates were dried overnight at room temperature and then stored at 4 C. Just prior to use, test wells were made and the test wells were filled with 5 µl of samples. Unless otherwise stated, the diameter of hemolytic zones was measured after the HI plates had been incubated at 37 C for 24 hrs.

**Growth, Alpha Toxin Production, and pH Development**

(a) **The organism.** The strain of *C. perfringens* used in the experiment was obtained from the Department of Animal Science, Ohio State University, Columbus, Ohio.

(b) **Test media.** Brewer Thioglycollate medium(Difco) was used as the test medium. Also beef broth(RJR Foods, Inc., Winston-Salem, North Carolina) was used as a base medium in which ground beef or/and Promine-D(Central Soya, Chicago, Illinois) were prepared as follows: the beef broth was blended with 20% ground beef, or the beef broth was blended with 14% ground beef and 6% Promine-D. Both of the products were prepared and sterilized(121 C for 15 min) in the blendor jars. They were blended well before use.

(c) **Measurement of growth and alpha toxin production.** One
tenth milliliter of an overnight broth culture of *C. perfringens* was inoculated into 40 ml of the test media contained in an Erlenmeyer (50 ml) screw cap flask. When the Thioglycollate medium was used as a growth medium, incubation was carried out at 15, 25, 35, 45, and 50 C in a temperature-controlled water bath. However, the culture was incubated at 37 C when the beef broth with ground meat and/or soy protein were used as the test media. Samples were taken at appropriate time intervals in sterile test tubes (12 x 100 mm) for the measurement of viable counts, alpha toxin production, and pH. The viable cell population was estimated by pour plates prepared with SFP (Shahidi and Ferguson, 1971) agar without the addition of Polymyxin B sulfate and Kanamycin sulfate. Alpha toxin production was measured by the HI test. pH was measured using PHM 62 Standard pH Meter (Radiometer Copenhagen).

(d) Treatment of growth data. Growth curves were constructed by plotting the logarithm of the colony forming units (CFU) versus incubation time. The generation time was calculated by the formula: \( G_t = \frac{t}{n} = \frac{t}{(3.3 \log_{10} b/a)} \)
where \( G_t \) (generation time) is equal to the time, \( t \), (the elapsed time between measurement of \( a \), the initial population, and \( b \), the final population), divided by the number of generations, \( n \) (number of generations being equal to 3.3 \( \log_{10} b/a \)).
Polyacrylamide Gel Electrophoresis

The electrophoretic technique employed in this study was a slight modification of the method described by Laemmli (1970). The whole gel consisted of two portions: stacking gel (upper gel) and separating gel (lower gel). The separating gels of 10% acrylamide was prepared by mixing 18 ml of acrylamide stock solution (22.2 g acrylamide + 0.6 g methylene bisacrylamide in a final volume of 100 ml), 10 ml of 1.5 M Tris-HCl buffer containing 0.4% SDS (Sodium dodecyl sulfate, pH 8.8), 1 ml of ammonium persulfate (10 mg dissolved in 1 ml of water), 11 ml of water and 0.03 ml TEMED (N, N, N', N'-tetramethylenediamine). Gels (6 cm) were prepared in 8 cm glass tubes with an inside diameter of 5 mm. After complete polymerization of the separating gels, the stacking gels (7 mm in length) were formed on the top of the separating gels. The stacking gels (3% acrylamide) were prepared by mixing 0.66 ml acrylamide stock solution, 1.25 ml of 0.5 M Tris-HCl buffer containing 0.4% SDS (pH 6.8), 0.1 ml of ammonium persulfate (10 mg per ml), 3 ml of water and 0.01 ml TEMED. After complete polymerization of the gels, 50 μl of the alpha toxin samples were applied on top of the gel with 10 μl of bromphenol blue (0.1%). The gel columns were then immersed in a bath buffer (pH 8.3) containing 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Electrophoresis was carried out with a current of 2 mA per gel and until the bromphenol
blue marker reached the bottom of the gel. After electrophoresis, the gels were removed from the tubes and stained overnight in test tubes at room temperature with 0.4% coomassie brilliant blue in 50% methanol and 9.2% acetic acid. The gels were rinsed and destained overnight in a solution containing 50% methanol and 9.2% acetic acid. After complete destaining, the gels were scanned using a Chromoscan Densitometer (Joyce and Loebl, Burlington, Mass.). An orange filter was used to give an approximate wavelength of 595 nm. Also, the 10 unstained gels after electrophoresis were cut and crushed into 5 fractions according to their relative mobility (Rm). The extracted fractions were suspended in distilled water. Half of each fraction was dialyzed overnight against distilled water at 4 C. The dialyzed and undialyzed fractions were utilized for further characterization.

(a) Determination of protein content. The protein content of the various fractions was determined by the modified Lowry method (Herbert, et al., 1971). The standard curve was prepared with crystalline bovine serum albumin.

(b) Measurement of phospholipase C activity. Phospholipase C activity was measured by a modified titrimetric method according to Zwaal, et al. (1971). One fresh egg yolk was suspended in 100 ml 0.15 M NaCl, supplemented with 10 m M CaCl₂, and centrifuged at 20,000 RCF for 40 min at 4 C. Two milliliters of this substrate was mixed with 8 ml of the NaCl
solution mentioned above, and 0.05 ml of the sample. The release of titratable H\(^+\) was followed in a titration unit ABU 12 in connection with a Titrigraph SBR\(\text{3}\) (Radiometer, Copenhagen, Denmark) by instantaneous addition of 0.01 M NaOH. The reaction mixture was kept at 37 C and pH 7.2. The mixture was continuously stirred with a teflon-coated magnetic stirrer and kept under nitrogen atmosphere. One unit of phospholipase C was defined as the amount of enzyme which liberated one micromole of titratable H\(^+\) per min.

(c) Measurement of hemolytic activity. The hemolytic activity of the fractions was determined by the use of HI test described earlier.

(d) Measurement of hemolytic activity on the HI plate containing horse red blood cell. HI plates were made with horse red blood cells following the same procedure described earlier for human red blood cells. The hemolytic activity of the fractions was determined using the horse rbc plates.

Heat Inactivation of Alpha Toxin

For heat inactivation, 0.1 ml aliquots of alpha toxin solution in small, thin-walled glass test tubes (length, 75 mm; inside diameter, 8 mm; wall thickness, 0.2 mm) were placed in an oil bath equilibrated at the desired temperature. The glass test tubes were cork-stoppered to prevent evaporation of the toxin solution. At various time intervals,
the test tubes were removed into an ice-water bath. For determining thermal inactivation time, 5 µl portions of the heated alpha toxin solution were spotted in the test wells of the HI plate, and the hemolytic activity was determined after 24 hrs incubation at 35 C.

Treatment of Data

For the methods of quantification of alpha toxin i.e. radial immunodiffusion(RID), lecithovitellin agar(LV) test, and hemolysin indicator plate(HI) test, standard curves were constructed by plotting the logarithm of the concentration of alpha toxin versus the diameter of reaction zone. The best straight line was fitted by the least square method (Topiwala, 1973).

In the heat inactivation study of alpha toxin, the D value, which represents the time necessary for the toxin concentration to be decreased by 90%, was estimated from the best fit line or by the equation: D = \( \frac{t}{\log b - \log a} \) where, \( t \) is the length of time in which the alpha toxin was heated, \( b \) is the toxin concentration at initial time, \( a \) is the toxin concentration remained after heating time.
RESULTS

Assay Procedures

At the onset, the study was concerned with an evaluation of a number of procedures for the quantification of the alpha toxin of *C. perfringens*. Some of these procedures are currently in use for alpha toxin assay; others were adapted from unrelated systems. For this phase of the study, the following methods were examined: electroimmunodiffusion (EID), radial immunodiffusion (RID), lecithovitellin agar (LV) test and Hemolysin indicator plate (HI) test. Commercial source (Wellcome Reagents Ltd, Beckenham, England) of alpha toxin and antiserum was used.

**Electroimmunodiffusion.** Initially, it was postulated that immunological techniques might be suitable for the assay of alpha toxin. One such technique examined was EID. The procedure followed was that described by Schanbacher and Smith (1974) and Sinha and Mikolajcik (1974). The following variables were studied: antiserum concentration levels of 1, 3 and 5%; electrophoresis times of 2 and 4 hrs; and alpha toxin concentrations of 0.2 to 25 unit/ml.

Although characteristic cone-shaped precipitin zones were obtained, these lacked the necessary clarity and sharp-
ness for accurate quantification. The reason for failure to obtain clear precipitin zones with the EID procedure was not explored beyond the variables listed above.

Attention was then directed to the application of another immunological procedure: the radial immunodiffusion technique (Mancini, et al., 1965).

**Radial immunodiffusion.** In the radial immunodiffusion test, migration of antigen (alpha toxin) is inhibited by antiserum (anti-alpha toxin). The higher the alpha toxin concentration, the larger precipitin ring formed. Also, the weaker the antiserum the farther the antigen must migrate before equilibrium is achieved.

A linear relationship between alpha toxin concentration in the range of 3.2 to 75 units/ml and the diameter of precipitin ring formed was observed when the data were plotted on a semilogarithmic scale (Fig. 1). The lower limits of detection of alpha toxin appeared to be approximately 3.2 units/ml.

Chemical tests were next evaluated for the quantification of alpha toxin: lecithovitellin agar test (Sheldon, et al., 1959) and hemolysin indicator plate test (Harmon and Kautter, 1970).

**Lecithovitellin agar.** Alpha toxin decomposes phospholipid complexes which occur as emulsifying agents in egg
Fig. 1. Quantification of alpha toxin from *C. perfringens* by the radial immunodiffusion technique. Data are based upon 6 trials. The linear regression equation is \( \log Y = 0.225X + 0.0109 \).
yolk. The enzymic activity of alpha toxin breaks the emulsion and liberates free fat so that turbidity is observed. Prior to use, the lecithovitellin plates are translucent, pale whitish-yellow in appearance. After the alpha toxin reacts with the lecithovitellin the reaction zones are homogeneous cream-colored areas of flocculation. As the unreacted areas of agar are somewhat opaque it is desirable to produce a system of a high contrast so that reaction zones can be accurately measured.

A linear relationship was obtained between the log of the alpha toxin concentration and the reaction zone diameter (Fig. 2). Below 0.2 unit of alpha toxin per ml, the zone diameter appears to vary randomly above and below the predicted values upon a direct extrapolation of the regression lines. Lack of contrast between reacted and unreacted lecithovitellin zones which resulted in reduced accuracy in measurement may have been involved. The contrast between the areas of reaction and the unreacted lecithovitellin was not sufficient to allow accurate measurement at lower(0.2 unit/ml) concentration of alpha toxin. An attempt to improve the contrast by manipulating egg yolk concentration in the range of 0.1 to 2% failed.

**Hemolysin indicator plate.** Alpha toxin attacks phospholipid complexes in the red blood cell membrane which is
Fig. 2. Quantification of the alpha toxin of *C. perfringens* by the lecithovitellin plate method. Data are based upon 6 trials. The linear regression equation is 
\[ \log Y = 0.129 X - 1.735. \]
$r = 0.972$
essential for the structural integrity of red blood cells. Thus, the reaction zones produced by alpha toxin on HI plates are clear areas of hemolysis easily differentiated from the red, translucent appearance of the unreacted agar. A linear relationship was also obtained between the alpha toxin concentration in the range of 0.0003 to 8.0 units/ml and the hemolytic zone diameter on a semilogarithmic scale with a broken point at approximately 0.01 units/ml (Fig. 3).

A comparison was made of the sensitivity of the three different alpha toxin assay methods studied (Table 2 and Fig. 4). The data reveal that the HI test was 10,000 times more sensitive than the RID or 633 times more sensitive than the LV test. Thus, among the techniques employed, the HI

Table 2. Comparison of concentration of alpha toxin required to produce a minimum detectable reaction zone diameter.

<table>
<thead>
<tr>
<th>Assay Method</th>
<th>Minimum detectable Reaction zone Diameter (mm)</th>
<th>Concentration of Alpha toxin (unit/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RID</td>
<td>3.0</td>
<td>3.2</td>
</tr>
<tr>
<td>LV</td>
<td>8.0</td>
<td>0.19</td>
</tr>
<tr>
<td>HI</td>
<td>3.0</td>
<td>0.0003</td>
</tr>
</tbody>
</table>
Fig. 3. Quantification of alpha toxin of C. perfringens by the HI plate method where Thioglycollate medium was used as diluent for the alpha toxin. Data are based upon 56 trials. The linear regression equation between 0.01-8 units/ml with a r of 0.998 is \[ \log Y = 0.179 X - 3.555. \]
Fig. 4. Sensitivity of the radial immunodiffusion (RID), lecithovitellin agar test (LV), and hemolysin indicator plate (HI) procedure for the quantification of the alpha toxin of Clostridium perfringens.
test was the most sensitive. Apparently, immunological techniques lack the sensitivity necessary for detection of extremely small amounts of alpha toxin.

Factors Affecting Hemolysin Indicator Test

Because the HI test was found to be the most sensitive assay method for alpha toxin among the techniques studied, attention was then directed to factors affecting its sensitivity. These included: nature of diluting agents, types of agar used, minerals, chelating agents, red blood cell concentration, and incubation temperature and time.

Diluting agents. The effect on the HI test of a physiological saline (0.85% NaCl) and a Brewer Thioglycollate medium (Difco, 0236) as diluting agents of alpha toxin is shown on Fig. 5 and 6.

The concentration of alpha toxin required for the minimum detectable size of hemolytic zone (approximately 3.0 mm) was 0.02 unit per ml when the toxin was diluted in saline. On the other hand, it was 0.0003 unit per ml when the Thioglycollate medium was used as a diluent of alpha toxin. Therefore, the sensitivity of HI test was 67 times greater when the alpha toxin was dissolved in a Thioglycollate medium rather than saline. However, the diluting agent effect on the sensitivity of HI test diminished at a concentration
Fig. 5. HI test of *C. perfringens* alpha toxin.
The volume of the toxin spotted was 5 µl.

A; (Top two rows)
alpha toxin in Thioglycollate medium
The concentrations of alpha toxin spotted were starting at the left, 0.003051, 0.006103, 0.01220, 0.02441, 0.03662 and 0.04882 unit/ml

B; (Bottom two rows)
alpha toxin in physiological saline
The concentrations of alpha toxin spotted were starting at the left, 0.003051, 0.006103, 0.01220, 0.02441, 0.03662 and 0.04882 unit/ml.
Fig. 6. The effect of the diluting agents used for alpha toxin on the HI test.
leucithinase conc., unit/ml

hemolytic zone, mm

10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^0 10^1

10 16 24

NaCl

THIOGLYCOLLATE MEDIUM
higher than 3.5 unit per ml.

Because Thioglycollate medium is a complex medium, individual components (Table 3) used in its formulation were tested as diluents for alpha toxin in order to ascertain their influence on the HI test. Results are shown in Fig. 7.

Table 3. Composition of the Brewer Thioglycollate medium (Difco, 0236)

<table>
<thead>
<tr>
<th>Ingredients per liter,</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef, infusion from</td>
<td>500 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Dipotassium phosphate</td>
<td>2 g</td>
</tr>
<tr>
<td>Proteose peptone, Difco</td>
<td>10 g</td>
</tr>
<tr>
<td>Bacto-dextrose</td>
<td>5 g</td>
</tr>
<tr>
<td>Sodium thioglycollate</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Bacto-methylene blue</td>
<td>0.002 g</td>
</tr>
<tr>
<td>Bacto-agar</td>
<td>0.5 g</td>
</tr>
</tbody>
</table>

Among the ingredients, only proteose peptone (Difco, 0120) showed the same size of hemolytic zone as was observed with Thioglycollate medium at the same alpha toxin concentration. It was postulated that the proteose peptone fraction of Thioglycollate medium was the major contributor to the increased sensitivity of the HI procedure, when the medium was used as a diluent for alpha toxin.
Fig. 7. Effect of glucose (G), $K_2HPO_4$ (K), sodium thioglycollate (T), beef extract (BE) and proteose peptone (PP) as diluting agents of alpha toxin on HI test.
lecithinase conc., unit/ml

hemolytic zone, mm

10^{-1}

10^{0}

10^{1}

10^{2}

10^{3}

10^{4}

G

PP

K

BE

T
Type of agar. Different commercial agars were compared as to their influence on the size of hemolytic zone on HI plates. The types of agar tested were Epiagar (Oxoid), Panagar (Oxoid), and Agar Agar No. 3 (Oxoid). The mineral composition of the agars are shown in Table 4. The alpha toxin was diluted in saline (Fig. 8) or in Thioglycollate medium (Fig. 9).

Table 4. Mineral composition of the different types of agar.

<table>
<thead>
<tr>
<th></th>
<th>Epiagar</th>
<th>Panagar</th>
<th>Agar Agar No. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>0.07%</td>
<td>0.05%</td>
<td>0.45%</td>
</tr>
<tr>
<td>Cu</td>
<td>1-4 ppm</td>
<td>2 ppm</td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>30 ppm</td>
<td></td>
<td>287 ppm</td>
</tr>
<tr>
<td>Mg</td>
<td>0.02%</td>
<td></td>
<td>0.24%</td>
</tr>
</tbody>
</table>

(Data supplied by Wilson Diagnostics, Inc., Glenwood, Ill.)

For either diluting system, Agar Agar No. 3 resulted in the largest hemolytic zone and Epiagar the smallest. The higher content of Ca and Mg in Agar Agar No. 3 might have contributed to the higher sensitivity of HI test. Calcium and magnesium have been demonstrated to activate alpha toxin (Zamecnik, et al., 1947; Ottolenghi, 1969; Ispolatovskaya, 1970). Again, at the same concentration of alpha toxin and irrespective of agars used, the hemolytic
Fig. 8. Effect of types of agar, Epi (Epiagar), Pan (Panagar), and Agar 3 (Agar Agar No. 3), used in preparation of HI plates on HI test. (Saline used as diluent of alpha toxin.)
lecithinase conc, unit/ml

hemolytic zone, mm

Epi
Pan
Agar 3
Fig. 9. Effect of types of agar, Epi (Epiagar), Pan (Panagar), and Agar 3 (Agar Agar No. 3), used in preparation of HI plates on HI test. (Thioglycollate medium used as diluent of alpha toxin)
zone diameter was greater when the alpha toxin was diluted in the Thioglycollate medium than saline.

**Minerals and sodium thioglycollate.** Calcium chloride was incorporated into the Epi-HI agar at the concentration of 0.3% and 1.4%. Data are shown in Fig. 10 where saline was diluent and in Fig. 11 for Thioglycollate medium as diluent.

Addition of 0.3% CaCl$_2$ in the Epi-HI agar sharply increased the size of hemolytic zone. However, a further increase in the concentration of CaCl$_2$ to 1.4% diminished the size of the hemolytic zone. In all instances, however, the zones were larger than those observed with the control.

The increase in the size of the hemolytic zone upon addition of CaCl$_2$ in the HI agar was not observed when CaCl$_2$ was incorporated into Agar Agar No. 3, presumably because Agar Agar No. 3 contains sufficient CaCl$_2$ (Table 4) to enhance the alpha toxin activity.

Incorporation of 0.3% MgCl$_2$ into the Agar Agar No. 3-HI agar increased the size of the hemolytic zone (Fig. 12 and 13). The activation of alpha toxin by magnesium has been reported by Zamecnik, *et al.* (1947). When 0.5% sodium thioglycollate (HS·CH$_2$·COONa) was incorporated into the HI agar plates prepared with Agar Agar No. 3, the size of the hemolytic zone was decreased when compared to the control (Fig. 12 and 13).
Fig. 10. Effect of 0.3 and 1.4% CaCl₂ incorporation in agar on HI test. (Saline as diluent for alpha toxin).
Fig. 11. Effect of 0.3 and 1.4% CaCl$_2$ incorporation in agar on HI test. (Thioglycollate medium as diluent for alpha toxin).
hemolytic zone [mm]

lecithinase conc [unit/ml]

10^{-4}  10^{-3}  10^{-2}  10^{-1}  10^0  10^1

0.3% CaCl₂

1.4% CaCl₂

control
Fig. 12. Effect of 0.5% sodium thioglycollate (Th) or 0.3% MgCl₂ incorporation in agar on HI test. (Saline as diluent for alpha toxin).
Leclthinase

Conc (Unit/ml)

Control

0.5% Th.

0.3% MgCl₂

Hemolytic Zone (mm)

Lecithinase Conc (Unit/ml)

10⁻¹

10⁻²

10⁻³

10⁻⁴

8

16

24
Fig. 13. Effect of 0.5% sodium thioglycollate (Th) or 0.3% MgCl₂ incorporation in agar on HI test. (Thioglycollate medium as diluent for alpha toxin).
Hemolytic Zone (mm)

Lecithinase Conc.(Unit/ml)

- Control
- 0.5% Th.
- 0.3% Mg Cl₂
Sodium thioglycollate has been reported to inhibit the alpha toxin activity possibly by reducing the disulfide linkages to sulfhydryl groups of alpha toxin molecule (Smith, 1968).

The increased sensitivity of the HI test when Thioglycollate medium was used as diluent was shown again.

Chelating agents. Addition of 0.01 M EDTA or 0.3% Na-citrate in Agar Agar No. 3-HI plates completely inhibited the hemolytic zone formation at even high concentrations (3 unit per ml) of alpha toxin. Chelating agents are known to tightly bind Ca and, thus, may inhibit alpha toxin activity.

Red blood cell (rbc) concentration. The red blood cell concentration of the HI plates was manipulated by counting the number of rbc in washed preparations using a hemocytometer chamber and then adjusting the concentration with different volumes of the saline agar base. Results shown in Fig. 14 and 15 indicate that the higher rbc concentration in the HI plate, the smaller hemolytic zone diameter, and vice versa.

Because phospholipids in red blood cell membrane may be utilized as a substrate for alpha toxin in HI test, it would be reasonable to expect that size of the hemolytic zone diameter would be inversely proportional to the concentration of rbc in the HI plates. Hence, the lower the rbc concentration, the greater the sensitivity where only the
Fig. 14. Effect of red blood cell concentration on HI test. Numbers refer to rbc concentration $x 10^6$/ml agar.
(Saline as diluent of alpha toxin).
Fig. 15. Effect of red blood cell concentration on HI test. Numbers refer to rbc concentration $\times 10^6/ml$ agar.

(Thioglycollate medium as diluent of alpha toxin).
hemolytic zone, mm

lecithinase conc. unit/ml
size of hemolytic zone was used as an index of sensitivity. However, the higher rbc concentration of HI plates was superior to lower rbc concentration in terms of clarity of hemolytic zone. At lower than $2.1 \times 10^6$ rbc per ml, the hemolytic zone was too indistinct to be measured precisely. Thioglycollate medium was demonstrated as a better diluting agent of alpha toxin than saline at the various concentrations of rbc studied.

**Incubation temperature and time.** The influence of incubation temperature of 21, 35 and 45 C, and incubation time of 6, 12, 24 and 48 hrs were studied for the sensitivity of the HI test.

The response of hemolytic zones was greater when the HI plates were incubated at 35 C than at 21 C(Fig. 16, 17, 18, and 19). With incubation at 45 C, the red blood cells deteriorated, and the readings on the hemolytic zone diameter could not be made. As to the effect of incubation time, longer incubation times yielded larger hemolytic zones(Fig. 16, 17, 18, and 19).

**Commercial preparations.** Alpha toxin of C. perfringens obtained from Wellcome Reagents Limited, Beckenham, England and from Calbiochem, San Diego, California was compared for the hemolytic activity(Fig. 20). According to Wellcome Reagents Limited, one unit of alpha toxin is based on the toxin/
Fig. 16. Effect of length of incubation of HI plates at 21 C on HI test.
(Saline as diluent of alpha toxin).
Fig. 17. Effect of length of incubation of HI plates at 35 C on HI test. (Saline as diluent of alpha toxin).
Fig. 18. Effect of length of incubation of HI plates at 21°C on HI test. (Thioglycollate medium as diluent of alpha toxin).
Fig. 19. Effect of length of incubation of HI plates at 35°C on HI test. (Thioglycollate medium as diluent of alpha toxin).
Fig. 20. Effect of different commercial sources of alpha toxin and different diluents used on the HI test.
(Calbiochem, A in saline and C in Thioglycollate medium; Wellcome, B in saline and D in Thioglycollate medium).
antitoxin neutralising test in mice injected intravenously. One unit of Calbiochem phospholipase C is defined as the activity liberating one micromole of inorganic phosphorus per minute at 37 °C, pH 7.3, with egg lecithin as the substrate. The use of different definitions for alpha toxin activity by the two different commercial sources also yielded different hemolytic zones on the basis of an equivalent unit per ml (Fig. 20). To achieve the same hemolytic zone, $1.215 \times$ the units of Calbiochem's alpha toxin were required for each unit of Wellcome's alpha toxin where saline was used as a diluent.

**Bacterial origins.** Both phospholipase C of *C. perfringens* and of *Bacillus cereus* obtained from Calbiochem, San Diego, California were compared for the hemolytic activity. For both, one unit was defined as the activity causing the liberation of one micromole of inorganic phosphorus per minute at 37 °C, pH 7.3 with egg lecithin as a substrate. For the same unit per ml, the phospholipase C of *C. perfringens* produced much a greater hemolytic zone than that of *B. cereus* (Fig. 21).

**Growth, Alpha Toxin Production, and pH Development of *C. perfringens***

The test media in which *C. perfringens* were grown are
Fig. 21. Comparison of lecithinase C from *B. cereus* (B.c.) and *C. perfringens* (C.p.) on HI test. Diluent was Thioglycollate medium (broken lines) or saline (solid lines).
as follows: (a) Brewer Thioglycollate medium (Difco, 0236),
(b) beef broth (RJR Foods, Inc., Winston-Salem, North Carolina) blended with 20% ground beef, and (c) the beef broth blended with 14% ground beef and 6% Promine-D (Central Soya, Chicago, Illinois).

In all instances, a 0.25% inoculum of overnight broth culture was used. The viable count was made by pour plates prepared with SFP base agar. Alpha toxin was quantified by the HI test.

**Thioglycollate medium.** The growth curves, alpha toxin production and pH development for *C. perfringens* in Thioglycollate medium at various incubation temperatures were determined.

Growth curves (Fig. 22-25) revealed that *C. perfringens* had the shortest generation time, i.e. grew fastest at 35°C (Fig. 23). At 45°C (Fig. 24), the growth was almost as fast as at 35°C, i.e. the difference in generation time between 35°C and 45°C was almost negligible (Table 5). Even at 50°C (Fig. 25), the growth was fairly rapid. At 25°C (Fig. 22), the growth was considerably slower than at 35°C. The time required to reach the stationary phase was also affected by the incubation temperature. It was approximately 4 to 5 hrs at 35, 45 and 50°C. In contrast, it was 22 hrs at 25°C.

At different incubation temperatures, the maximum viable
Fig. 22. Growth, pH, and alpha toxin production of *C. perfringens* in Thioglycollate medium at 25°C.
Fig. 23. Growth, pH, and alpha toxin production of *C. perfringens* in Thioglycollate medium at 35 C.
log CFU/ml

Time, days

pH

Alpha toxin, unit/ml

Log CFU/ml versus time, days graph. The graph shows the changes in log CFU/ml over time. The pH and alpha toxin levels are also indicated on the graph.
Fig. 24. Growth and alpha toxin production of C. perfringens in Thioglycollate medium at 45°C.
Fig. 25. Growth, pH, and alpha toxin production of *C. perfringens* in Thioglycollate medium at 50 °C.
The graph shows the relationship between time (days) and several parameters: Log CFU/ml (cells), pH, and alpha toxin (units/ml). The graph indicates a decrease in Log CFU/ml, pH, and alpha toxin over time.
Table 5. Generation time, Gt; maximum yield of alpha toxin, Y; and time required for 90% reduction of alpha toxin, Dt, in Thioglycollate medium at different incubation temperatures.

<table>
<thead>
<tr>
<th>Incubation Temperature (°C)</th>
<th>Gt (min)</th>
<th>Y (unit/ml)</th>
<th>Dt (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>61.5</td>
<td>4.47 x 10^{-2}</td>
<td>43.1</td>
</tr>
<tr>
<td>35</td>
<td>19.3</td>
<td>2.24 x 10^{-1}</td>
<td>6.3</td>
</tr>
<tr>
<td>45</td>
<td>19.7</td>
<td>5.63 x 10^{-3}</td>
<td>60.3</td>
</tr>
<tr>
<td>50</td>
<td>22.6</td>
<td>3.55 x 10^{-3}</td>
<td>121.2</td>
</tr>
</tbody>
</table>

The population reached at the end of logarithmic growth phase also varied. It was approximately $2.2 \times 10^8$ organisms per ml at 25, 35 and 45 °C and $1.3 \times 10^7$ organisms per ml at 50 °C. At 15 °C, the lowest temperature studied, *C. perfringens* died off. A two-log cycle reduction in viable count occurred in 6 hrs. In general, after the stationary phase the higher the incubation temperature, the more rapid the decline phase.

The optimum temperature for alpha toxin production was 35 °C (Fig. 23). Also, the rate of alpha toxin production was closely related to the growth curve, irrespective of the incubation temperature. However, the maximum yield of alpha toxin did not correlate well with the growth rate. For exam-
pie, the growth rate at 35°C was practically the same as that at 45°C (Fig. 24), whereas the maximum yield of alpha toxin at 35°C was approximately 40 times greater than at 45°C (Table 5). The stability of alpha toxin was greatly dependent on the temperature of incubation. The destruction rate of alpha toxin closely obeyed the first order kinetics. Alpha toxin was most unstable at the incubation temperature of 35°C.

In general, pH of the medium dropped when maximum growth and alpha toxin production was attained (Fig. 22-25). The optimum pH for alpha toxin activity and production was reported to be approximately 7.0-7.2 by Pivnick, et al. (1965). Therefore a drop in pH to 5.0 could be one of the limiting factors for alpha toxin production.

**Beef broth with ground beef and/or Promine-D.** Before proceeding to a study of alpha toxin production by *C. perfringens* in beef broth with ground beef and/or Promine-D, recovery of known amounts of alpha toxin suspended in beef broth was determined. The recovery was essentially 100%, i.e. quantification of alpha toxin using beef broth as diluent yielded practically the equivalent hemolytic zone diameter as that observed where Thioglycollate medium was the diluent.

When *C. perfringens* was inoculated in beef broth (RJR Foods, Inc., Winston-Salem, North Carolina) with 20% ground
beef and incubated at 35 C, the organism grew slower and produced less alpha toxin than when it was grown in Thioglycollate medium (Fig. 26 and Table 6).

Table 6. Comparison of generation time, $G_t$, and maximum alpha toxin yield, $Y$, in beef broth and 20% ground beef, and Thioglycollate medium at incubation temperature of 35 C.

<table>
<thead>
<tr>
<th>Media</th>
<th>$G_t$ (min)</th>
<th>$Y$ (unit/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef broth-plus 20% ground beef</td>
<td>156</td>
<td>$8.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>Thioglycollate medium</td>
<td>19.3</td>
<td>$2.24 \times 10^{-1}$</td>
</tr>
</tbody>
</table>

However, when beef broth blended with 14% ground beef and 6% Promine-D (Central Soya, Chicago, Illinois) was used as a medium at 35 C, _C. perfringens_ failed to grow. Instead, a two log cycle reduction in viable counts was observed in 24 hrs.

Population levels of _C. perfringens_ and amount of alpha toxin produced. The relationship between the viable count of _C. perfringens_ on SFP base agar and the amount of alpha toxin produced at various incubation temperatures is presented in Fig. 27. At a population level of $10^8$, _C. perfringens_
Fig. 26. Growth and toxin production of *C. perfringens* at 35 C in beef broth plus 20% ground beef (solid line) or beef broth plus 14% ground beef and 6% Promine-D (dotted lines).
Fig. 27. Relationship between viable counts of *C. perfringens* and alpha toxin production in Thioglycollate medium at different incubation temperatures.
produced approximately $1.0 \times 10^{-1}$ unit/ml of alpha toxin at 35°C and $6.5 \times 10^{-3}$ unit/ml at 45°C. It is apparent that incubation temperature will influence the amount of alpha toxin produced.

The relationship between substrate, population levels and the amount of alpha toxin produced was also studied (Fig. 28). For example, at a population level of $10^7$, *C. perfringens* produced approximately $7.1 \times 10^{-3}$ unit/ml of alpha toxin in Thioglycollate medium and $4.0 \times 10^{-4}$ unit/ml in beef broth blended with 20% ground beef.

Therefore, when population levels of *C. perfringens* are to be estimated from the amount of alpha toxin detected from a system, a knowledge of incubation temperature and time, and of the growth medium is essential.

**Selected Chemical and Physical Properties**

Two commercial preparations of alpha toxin were resolved by polyacrylamide gel (PAG) electrophoresis. The fractions obtained upon PAG electrophoresis were further characterized by densitometer scanning, protein determination, phospholipase C assay and hemolytic activity quantification. Hemolytic activity was quantified on HI plates made with both human and horse rbc.

The thermal inactivation characteristics of alpha toxin
Fig. 28. Relationship between viable counts of *C. perfringens* and alpha toxin production in Thioglycollate medium(TM) and beef broth with 20% ground beef(BB).
in saline and a Thioglycollate medium were studied at 55 to 95 C. Individual components used in the commercial preparation of Thioglycollate medium and the fractions of Thioglycollate medium obtained by molecular sieving were also utilized as heating menstrua for alpha toxin.

Polyacrylamide gel electrophoresis. Polyacrylamide disc gel electrophoresis was run in an alkaline system with a slight modification of the procedure of Laemmli (1970), using Tris-glycine buffer with 0.1% SDS, pH 8.3, and a gel concentration of 10%.

The electrophoretic patterns shown in Fig. 29 reveal that the alpha toxin preparation obtained from Wellcome Reagents Ltd. (Beckenham, England) contained at least 7 distinct protein bands. At least 4 distinct protein bands were observed with alpha toxin obtained from Calbiochem, San Diego, California.

The densitometer scans obtained from the electrophoretogram are shown in Fig. 30 and 31. The area under the densitogram of each fraction was tabulated (Table 7). These revealed that for both Wellcome and Calbiochem alpha toxin, Fraction 5 exhibited the largest area under the densitogram.

A duplicate unstained gel was placed next to a stained gel in order to locate areas of interest. The unstained gels
Fig. 29. Polyacrylamide disc gel electrophoresis of alpha toxin of *C. perfringens*

Tubes A and B: alpha toxin of Wellcome Reagents Ltd., Beckenham, England

Tubes C and D: alpha toxin of Calbiochem, San Diego, California.
Fig. 30. Densitometer scans obtained from the electrophoretogram of Wellcome alpha toxin.
Fig. 31. Densitometer scans obtained from the electrophoretogram of Calbiochem alpha toxin.
Table 7. Selected characteristics of fractions isolated by polyacrylamide gel electrophoresis of two different commercial preparations of alpha toxin.

<table>
<thead>
<tr>
<th>Commercial source</th>
<th>Fraction No.</th>
<th>Relative mobility (Rm)</th>
<th>Area under densitogram (mm²)</th>
<th>Total Protein content (ug)</th>
<th>Hemolytic activity (unit)</th>
<th>Specific Hemolytic activity (unit/mg protein)</th>
<th>Total activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wellcome</td>
<td>1*</td>
<td>5.0</td>
<td>24</td>
<td>0.0000</td>
<td>0.033</td>
<td>21.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.10-0.29</td>
<td>32</td>
<td>0.0100</td>
<td>0.312</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.29-0.56</td>
<td>172</td>
<td>3.3560</td>
<td>19.512</td>
<td>95.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.56-1.00</td>
<td>342</td>
<td>0.0556</td>
<td>0.163</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Calbiochem</td>
<td>1*</td>
<td>5.0</td>
<td>8</td>
<td>0.0048</td>
<td>0.600</td>
<td>7.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.22-0.36</td>
<td>132</td>
<td>0.0520</td>
<td>0.394</td>
<td>5.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.36-0.56</td>
<td>96</td>
<td>0.4000</td>
<td>4.167</td>
<td>57.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.56-1.00</td>
<td>192</td>
<td>0.0036</td>
<td>0.019</td>
<td>0.27</td>
<td></td>
</tr>
</tbody>
</table>

* Stacking gel
were cut into 5 fractions according to their relative mobility (Rm). The Rm range of the areas cut was: for Wellcome alpha toxin, 0-0.10, 0.10-0.29, 0.29-0.56, and 0.56-1.0 (Fig. 32) and for Calbiochem alpha toxin, 0-0.22, 0.22-0.36, and 0.36-0.56 and 0.56-1.0 (Fig. 33). Stacking gels were also included in the fraction. The cut portions from the gels were suspended in distilled water. Half of each fraction was dialyzed against distilled water at 7°C overnight. The dialyzed and undialyzed fractions were then characterized for protein content, phospholipase C activity by the titrometric procedure and hemolytic activity on HI plates made with both human and horse rbc.

The hemolytic activity of each fraction is shown in Table 7 and in Fig. 32 and 33. Protein content of each fraction determined by modified Lowry method (Herbert, et al., 1971) is also shown in Table 7. The nondialyzed fractions produced greater hemolytic zones than the dialyzed fractions probably due to the presence of 0.1% SDS in the running buffer which exhibited a slight hemolytic activity. Hence, quantification of hemolytic activity was based on the dialyzed fractions. For Wellcome's alpha toxin, Fraction 5 with a Rm of 0.56-1.0 contained the highest amount of protein whereas Fraction 4 with a Rm of 0.29-0.56 contained one half the protein of Fraction 5. Fraction 4, however, con-
Fig. 32. Hemolytic test of non-dialyzed (ND) and dialyzed (D) fractions obtained upon polyacrylamide gel electrophoresis of Wellcome's alpha toxin preparation. The arrows from the HI zones point to the specific areas of the gel from which the fraction was obtained.
Fig. 33. Hemolytic test of non-dialyzed (ND) and dialyzed (D) fractions obtained upon polyacrylamide gel electrophoresis of Calbiochem's alpha toxin preparation. The arrows from the HI zones point to the specific areas of the gel from which the fraction was obtained.
tained 95.6% of the total hemolytic activity. For the Calbiochem preparation, Fraction 4 with an Rm of 0.36-0.56 contained 58.49% of the hemolytic activity, with Fraction 3 containing 27.29% of the activity. Thus, a somewhat less clear separation on PAG was obtained with the Calbiochem preparation than the Wellcome alpha toxin.

Horse red blood cells are known to be sensitive to lysis by theta toxin of C. perfringens, but resistant to lysis by alpha toxin. Each PAG fraction failed to show any hemolytic activity on the HI plate made from horse rbc, suggesting that the fractions from PAGE of alpha toxin contained little theta toxin, if any. Only a high concentration of an untreated commercial alpha toxin preparation exhibited a slight hemolytic activity on the horse rbc-HI plate. This zone is outlined (inner square) on Fig. 34. This would indicate that the commercial alpha toxin may contain a slight amount of theta toxin. Storage of horse rbc-HI plate, spotted with commercial alpha toxin, for one week at 7 C following initial incubation of the plates for 24 hrs at 37 C produced a slight faint hemolytic zone (Fig. 35).

For both preparations, the lecithinase C assay by the pH stat procedure (Zwaal et al., 1971) revealed that only Fraction 4 exhibited phospholipase C activity. Therefore, it is apparent that a major portion of the alpha toxin is
Fig. 34. Hemolytic test of alpha toxin of C. perfringens on the horse rbc-MI plate. The plate was stored at 7°C for 24 hrs following initial incubation at 37°C for 24 hrs.

A; Wellcome's alpha toxin in physiological saline, 3.12 unit/ml,

B; Calbiochem's alpha toxin in physiological saline, 6.25 unit/ml, and

C; Supernatant of 10 hr (35°C) Thioglycollate medium culture of C. perfringens.
Fig. 35. Hemolytic test of alpha toxin of *C. perfringens* on the horse rbc-HI plate. The plate was stored for a week at 7°C following initial incubation of the plate for 24 hrs at 37°C.

A; alpha toxin in Thioglycollate medium
The concentrations of alpha toxin spotted were, from right to left, 0.0004, 0.0015, 0.0061, 0.0244, 0.0976, 0.3906 and 1.5625 unit/ml, and

B; alpha toxin in physiological saline
The concentration of alpha toxin spotted were, from right to left, 0.0004, 0.0015, 0.0061, 0.0244, 0.0976, 0.3906 and 1.5625 unit/ml.
present in the protein band with a Rm of 0.42 for Wellcome's preparation and in two protein bands (Rm, 0.31 and 0.42) for Calbiochem's preparation.

Heat inactivation. Physiological saline and Thioglycollate medium (Difco) were used as heating menstrua for alpha toxin during initial stages of the study. Heating temperatures selected were 55, 65, 75, 85, and 95°C.

Heat inactivation rates of alpha toxin in saline and Thioglycollate medium are shown in Fig. 36. The percentage of alpha toxin activity remaining was calculated by assigning the unheated control the value of 100. The inactivation rate of alpha toxin in both systems was linear with heating time on a semilogarithmic scale, i.e., the rate of loss of activity of alpha toxin closely followed first order kinetics in the range of 55 to 95°C for 18 min.

The "D" values (Table 8) when alpha toxin was heated in a physiological saline or a Thioglycollate medium at 55, 65, 75, 85, and 95°C were 25.5, 8.6, 7.4, 7.0 and 6.7 min, and 89, 57.5, 63.0, 78.5 and 75.0 min, respectively.

A striking feature of the heat inactivation characteristics of alpha toxin was that the "D" values were much greater at all temperature levels studied when the toxin was heated in Thioglycollate medium than in saline. In other words, alpha toxin was much more heat resistant in Thioglycollate
Fig. 36. Percentage of alpha toxin remaining after heating at temperature of 55-95 C in physiological saline (solid lines) and in Thioglycollate medium (broken lines).
Table 8. "D" values for alpha toxin in physiological saline and Thioglycollate medium.

<table>
<thead>
<tr>
<th>Heating temperature (°C)</th>
<th>Saline</th>
<th>Thioglycollate medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>25.5</td>
<td>89.0</td>
</tr>
<tr>
<td>65</td>
<td>8.6</td>
<td>57.5</td>
</tr>
<tr>
<td>75</td>
<td>7.4</td>
<td>63.0</td>
</tr>
<tr>
<td>85</td>
<td>7.0</td>
<td>78.5</td>
</tr>
<tr>
<td>95</td>
<td>6.7</td>
<td>75.0</td>
</tr>
</tbody>
</table>

medium than in saline. The increased heat resistance of alpha toxin in Thioglycollate medium is also supported by data shown in Table 9. For example, after heat treatment of 18 min at 95 °C, the percentage of activity of alpha toxin which remained was 0.18 in physiological saline and 53 in Thioglycollate medium.

A thermal inactivation time curve was prepared by plotting the log of D against the corresponding temperature. When the alpha toxin was heated in saline(Fig. 37), the decrease in "D" value at temperatures higher than 65 °C was surprisingly small. The broken curve below and above 65 °C in thermal inactivation time curve indicates that alpha toxin
Fig. 37. Thermal inactivation time curve of alpha toxin in physiological saline and Thioglycollate medium.
D value (min)

thioglycollate medium

0.85% saline

D value (min)

temperature (°C)
Table 9. The percent residual activity of alpha toxin when heated in physiological saline and Thioglycollate medium for 18 min.

<table>
<thead>
<tr>
<th>Heating temperature (°C)</th>
<th>Saline % Activity remaining</th>
<th>Thioglycollate medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>18.8</td>
<td>62</td>
</tr>
<tr>
<td>65</td>
<td>0.64</td>
<td>48</td>
</tr>
<tr>
<td>75</td>
<td>0.33</td>
<td>50</td>
</tr>
<tr>
<td>85</td>
<td>0.28</td>
<td>58</td>
</tr>
<tr>
<td>95</td>
<td>0.18</td>
<td>53</td>
</tr>
</tbody>
</table>

became more resistant to inactivation at temperatures higher than 65 °C. The "D" value was 21 min in the range of 55 to 65 °C and 252 min in the range of 65 to 95 °C.

When the alpha toxin was heated in Thioglycollate medium (Fig. 37), the thermal inactivation time curve exhibited a very unusual shape. In a normal thermal inactivation time curve of an enzyme or a microorganism, the curve will form a certain negative slope depending on the decrease in "D" value at a corresponding increment of heating temperature. However, in this case, the curve failed to show a definite negative slope suggesting an abnormal heat resistance beha-
vior for the alpha toxin in Thioglycollate medium.

Since the alpha toxin was more heat resistant in Thioglycollate medium than in saline, the individual commercially available components of the Thioglycollate medium (Table 3) were examined as heating menstrua for alpha toxin. The rate of inactivation of alpha toxin in the individual components of a Thioglycollate medium are shown in Fig. 38 for glucose, Fig. 39 for Na-thioglycollate, Fig. 40 for beef extract, Fig. 41 for K$_2$HPO$_4$ and Fig. 42 for proteose peptone. The data are tabulated in Table 10.

Table 10. The percent residual activity at 18 min of alpha toxin when heated in the individual components of Thioglycollate medium.

<table>
<thead>
<tr>
<th>Heating menstrua</th>
<th>Heating temperature(C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>55</td>
</tr>
<tr>
<td>0.5% glucose</td>
<td>93</td>
</tr>
<tr>
<td>0.05% Na-thioglycollate</td>
<td>80</td>
</tr>
<tr>
<td>0.6% beef extract</td>
<td>97</td>
</tr>
<tr>
<td>0.2% K$_2$HPO$_4$</td>
<td>3.6</td>
</tr>
<tr>
<td>1% proteose peptone</td>
<td>93</td>
</tr>
</tbody>
</table>

In most of the heating systems studied, rate of heat
Fig. 38. Percentage of alpha toxin remaining after heating at temperature of 55-95°C in 0.5% glucose.
Fig. 39. Percentage of alpha toxin remaining after heating at temperature of 55-95°C in 0.05% sodium thioglycollate.
Fig. 40. Percentage of alpha toxin remaining after heating at temperature of 55-95 °C in 0.6% beef extract.
Fig. 41. Percentage of alpha toxin remaining after heating at temperature of 55-95 C in 0.2% K$_2$HPO$_4$. 
Fig. 42. Percentage of alpha toxin remaining after heating at temperature of 55-95°C in 1% proteose peptone.
inactivation was linear with time. However, differences in amount of alpha toxin inactivated were observed. For example, the percentage of residual alpha toxin activity after heating for 18 min at 95°C in 0.5% glucose, 0.05% Na-thioglycollate, 0.6% beef extract, 0.2% K$_2$HPO$_4$ and 1% proteose peptone was 0.1, 4.6, 3.9, 0.1 and 93%, respectively. Thus, it was noted that alpha toxin was most heat stable in the proteose peptone (Fig. 43), with intermediate heat stability in beef extract or Na-thioglycollate (Fig. 43) and least stable in glucose or K$_2$HPO$_4$.

Attention was now directed to the fractionation of Thioglycollate medium by molecular sieving. The Diaflo ultrafiltration membrane (Amicon, Lexington, Mass.) was used for this purpose. Using Diaflo membranes of UM2, UM10, PM10, and PM30, fractions exhibiting the following molecular weights were obtained from Thioglycollate medium: MW>30,000, 30,000>MW>10,000, 10,000>MW>1,000, and 1,000>MW.

Alpha toxin was heated in each of these fractions. The percentage of alpha toxin activity remaining after heating at various temperatures is shown in Fig. 44-47. The percentage of alpha toxin activity remaining after heating for 18 min at 95°C in MW<1,000, 1,000<MW<10,000, 10,000<MW<30,000 and MW>30,000 was 3.2, 3.6, 31 and 62%, respectively (Table 11). Thus, it was noted that alpha toxin exhibited
Hemolytic test of alpha toxin after heating time (T) of 0, 3, 6, 9, 12, 15, 18 min at 95 °C. The heating menstrua were PP (proteose peptone) and BE (beef extract). The concentration of alpha toxin in 5 µl spotted at 0 min was 0.3906 unit/ml.
Fig. 44. Percentage of alpha toxin remaining after heating at temperature of 55-95 C in the fraction of MW<1,000 obtained from Thio-glycollate medium.
Fig. 45. Percentage of alpha toxin remaining after heating at temperature of 55-95 C in the fraction of $1,000 < \text{MW} < 10,000$ obtained from Thioglycollate medium.
Fig. 46. Percentage of alpha toxin remaining after heating at temperature of 55-95°C in the fraction of $10,000 < \text{MW} < 30,000$ obtained from Thioglycollate medium.
Fig. 47. Percentage of alpha toxin remaining after heating at temperature of 55-95°C in the fraction of MW>30,000 obtained from Thioglycollate medium.
maximum heat resistance in the fraction with a MW > 30,000, and minimum heat resistance in the fraction of MW < 1,000.

Table 11. The percent residual activity at 18 min of alpha toxin heated in fractions of different molecular weights separated from Thioglycollate medium.

<table>
<thead>
<tr>
<th>Heating menstrum</th>
<th>55</th>
<th>65</th>
<th>75</th>
<th>85</th>
<th>95</th>
<th>...% Activity remaining ..........</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW &lt; 1,000</td>
<td>72</td>
<td>8.5</td>
<td>6.2</td>
<td>5.3</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>1,000 &lt; MW &lt; 10,000</td>
<td>61</td>
<td>8.0</td>
<td>7.8</td>
<td>7.6</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>10,000 &lt; MW &lt; 30,000</td>
<td>80</td>
<td>22</td>
<td>16</td>
<td>20</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>30,000 &lt; MW</td>
<td>82</td>
<td>58</td>
<td>56</td>
<td>90</td>
<td>62</td>
<td></td>
</tr>
</tbody>
</table>

The "D" values (Table 12) for fraction of MW > 30,000 and MW < 1,000 were 361 and 140 min at 55 C, 81.6 and 17.1 min at 65 C, 72.3 and 15.4 min at 75 C, 843 and 14.4 min at 85 C, and 90.4 and 12.4 min at 95 C, respectively.

The log of "D" values was plotted against the corresponding temperature to construct a thermal inactivation curve. When alpha toxin was heated in the fraction of MW > 30,000, the thermal inactivation time curve (Fig. 48) was similar to that for Thioglycollate medium shown in Fig. 37.
Fig. 48. Thermal inactivation time curve of alpha toxin in the fractions of different molecular weights separated from Thioglycollate medium.
Table 12. "D" values of alpha toxin when heated in the fractions obtained through molecular sieving of Thioglycollate medium.

<table>
<thead>
<tr>
<th>Heating temperature (°C)</th>
<th>MW&lt;1,000</th>
<th>MW&lt;10,000</th>
<th>MW&lt;30,000</th>
<th>MW&gt;30,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>140</td>
<td>90.4</td>
<td>81.6</td>
<td>361</td>
</tr>
<tr>
<td>65</td>
<td>17.1</td>
<td>16.5</td>
<td>28.4</td>
<td>81.6</td>
</tr>
<tr>
<td>75</td>
<td>15.4</td>
<td>16.5</td>
<td>23.4</td>
<td>72.3</td>
</tr>
<tr>
<td>85</td>
<td>14.4</td>
<td>16.5</td>
<td>26.1</td>
<td>84.3</td>
</tr>
<tr>
<td>95</td>
<td>12.4</td>
<td>12.9</td>
<td>35.6</td>
<td>90.4</td>
</tr>
</tbody>
</table>

Again it was verified that alpha toxin was most stable in the fraction of MW>30,000. Therefore, it is postulated that a molecular species greater than 30,000 present in proteose peptone protects the alpha toxin against heat inactivation. The exact nature of this agent and the mechanism(s) by which it protects the alpha toxin against heat destruction await further study.

When alpha toxin was heated in the fraction of MW<1,000 or 1,000<MW<10,000(Fig. 48), the thermal inactivation time curve was similar to that for physiological saline shown in Fig. 37. Thus, alpha toxin, when heated in the fraction of
MW<1,000 or 1,000<MW<10,000, was shown to become more heat resistant above 65 C, an effect observed earlier in saline.

Our attention was next directed to the heat inactivation characteristics of alpha toxin produced by our strain of *Clostridium perfringens* when grown in a laboratory medium. *Clostridium perfringens* was grown in Thioglycollate medium for 10 hrs at 35 C using a 1% overnight culture as an inoculum. The culture was then harvested, centrifuged for 15 min at 3,000 RCF and the supernatant was heated at various temperatures. The rate of heat inactivation of the alpha toxin is shown in Fig. 49. Except during heating at 55 or 65 C, the inactivation curves show an inflection point at approximately 1-2 min heating time suggesting that there exists in the alpha toxin of the culture supernatant a small fraction with an increased heat resistance. Also, the alpha toxin from the culture supernatant was more resistant at 65 C than 95 C. A similar phenomenon was observed by Smith and Gardner(1950) when they heated alpha toxin in 0.01M CaCl₂. However, when commercial alpha toxin preparation was heated in 0.01M CaCl₂ in our laboratory, such a phenomenon could not be observed; a normal heat inactivation curve was observed(Fig. 50). In this regard, Nakamura, et al. (1969) reported that they also could not completely confirm the anomalous heat inactivation characteristic of alpha
Fig. 49. Percent residual activity of alpha toxin of culture supernatant of *C. perfringens* after heating at temperature of 55-95°C.
Fig. 50. Percentage of alpha toxin remaining after heating at temperature of 55-95°C in 0.01 M CaCl₂.
toxin observed by Smith and Gardner. These discrepancies may be explained by the postulation that different molecular forms of alpha toxin exist, or that different strains produce different phospholipase C enzymes.
DISCUSSION

Proliferation and alpha toxin production by Clostridium perfringens are closely related. It should therefore be possible to approximate the number of C. perfringens in a system by determination of the amount of alpha toxin present. To achieve this objective, at the outset it is necessary to understand the many factors affecting (a) the assay procedure, (b) toxin stability under handling, processing, and storage conditions, and (c) growth and toxin production of C. perfringens in laboratory and commercial food systems.

An ideal assay method for C. perfringens alpha toxin must be simple, specific, sensitive, quantitative, inexpensive and rapid. In terms of sensitivity, the hemolysin indicator plate (HI) method was most promising although it may lack the specificity of the radial immunodiffusion (RID) technique.

The RID procedure is based upon the formation of an antigen-antibody complex in the presence of specific anti-alpha toxin serum. Immunological techniques are known to be extremely specific and sensitive. However, our findings indicate that the HI procedure, a chemical, non-immunological method, was far more sensitive than RID. The reason(s) for the reduced sensitivity of the RID is not known. It is pos-
tulated that the antisera was of too weak strength or quality of antibody was too poor to form clear precipitin.

With respect to specificity of the HI method and possible interference by hemolysins produced by organisms other than *C. perfringens*, there are certain limitations. Many bacteria are known to produce hemolysins (Bernheimer, 1970). These include, to name a few, staphylococcal alpha toxin, staphylococcal beta hemolysin, streptolysin O, and *Clostridium hemolyticum* lysin. Therefore, care must be exercised when the HI test is conducted with an unknown food sample. Any positive hemolytic activity observed with a suspected food sample would provide only presumptive evidence that the food product at some time during processing or handling had been heavily contaminated with *C. perfringens*. Further confirmatory tests would be necessary before a definite conclusion could be made whether or not the suspected food product had been contaminated with *C. perfringens*. Here the RID test could confirm the specificity of the alpha toxin (hemolysin).

As another part of our study, it was shown that phospholipase C of *B. cereus* was much less hemolytic than that of *C. perfringens* on the same concentration basis of the enzyme. The difference of hemolytic activity may be explained by the different substrate specificities of the two enzymes as evidenced by van Deenen, et al. (1961) and Kleiman and Lands.
Sphingomyelin has been reported to be attacked by phospholipase C of *C. perfringens* (Matsumoto, 1961; Pastan, et al., 1968), but not by phospholipase C of *B. cereus* (Kleiman and Lands, 1969). Besides difference in substrate specificities of the two enzymes, these enzymes differ in immunological specificity, too, which was confirmed by us and others (Bonventre and Johnson, 1970).

In addition to being found in *C. perfringens* and *B. cereus*, lecithinase C has also been found in *Clostridium sordelli* (Miles and Miles, 1947), *Clostridium hemolyticum* (Macfarlane, 1948), *Pseudomonas aeruginosa* (Kurioka and Liu, 1967) and *Pseudomonas fluorescens* (Doi and Nojima, 1971). The lecithinase C of *C. sordelli* has been shown to have significantly less hemolytic activities than the lecithinase C of *C. perfringens* (Miles and Miles, 1947). Whether or not various phospholipase C enzymes mentioned above have the same substrate specificity, immunological specificity and other common characteristics remains to be seen.

The fact that the greater hemolytic response was obtained when the alpha toxin was dissolved in Thioglycollate medium than physiological saline suggests that a type of dispersing agent of alpha toxin must be taken into consideration before the hemolytic activity of the system is studied. This fact also questions the validity of common practices.
that have used physiological saline or simple buffer solution as a carrier of alpha toxin in a toxicological study using animals as subject.

The types of agar used for the preparation of HI plates were found to influence the sensitivity of the HI test. It is apparent that a certain activator or inhibitor in the components of agar will affect the sensitivity of the HI test. Agar Agar No. 3 containing higher contents of Ca and Mg than Epiagar or Panagar resulted in greater hemolytic zones. Addition of 0.3% CaCl₂ in the Epi-HI agar sharply increased the sensitivity of the HI test. However, 0.01M EDTA or 0.3% sodium citrate when incorporated in Agar Agar No. 3 completely inhibited formation of the hemolytic zone possibly by binding Ca and inhibiting alpha toxin activity. These results point out the importance of the type of agar used in the preparation of the HI plates and of its ultimate effect on the sensitivity of the procedure.

As the concentration of rbc in the HI plate decreased, the size of hemolytic zones was increased for an equal concentration of alpha toxin, and vice versa. Therefore, the rbc concentration in the HI plates must be decreased as low as possible in order to increase the sensitivity of alpha toxin detection from an unknown sample. However, because the hemolytic zone becomes too indistinct to be measured
precisely below a critical concentration ($2.1 \times 10^6$ rbc per ml of HI plate), a compromise should be reached between a sensitivity of the detection method and concentration of rbc used in the preparation of HI agar.

Another factor deserving of consideration is the source of the rbc. It is evident that horse rbc will not respond to alpha toxin and thus can not be used for the assay procedure. With respect to rbc of other species, those of rabbits and sheep are moderately susceptible whereas those of goats are comparatively resistant to alpha toxin (Smith and Holdeman, 1968).

Another factor influencing the sensitivity of HI test is length of incubation of the plates. It is apparent that the longer the incubation, the farther the alpha toxin will diffuse in the HI plate and the greater the contact between a substrate (rbc) and an enzyme (alpha toxin). Thus, the sensitivity of HI test will be increased. However, a shorter incubation time would be more advantageous for a microbiological control laboratory.

Temperature plays an essential role in bacterial growth and in toxin production. Cooking temperature will kill vegetative cells of *C. perfringens* but may cause heat activation of any spores present in a food. During cooling of large quantities of cooked food, as may occur in institutional kitchens, and during subsequent heating before serving,
food is exposed to a gradient of temperature where a rapid multiplication of heat activated spores can take place. The generation time of this organism is only 19 min in the temperature range of 35 to 45°C. In a study to simulate a typical food service operation, Tumoi et al. (1974) demonstrated that a ground beef gravy cooled by refrigeration was in the temperature range (31 to 50°C) for a rapid growth of *C. perfringens* for 2 hrs. Thus, during the time that gravy remained at a temperature in the optimum growth zone, multiplication could start at a high rate.

Although, our strain had a generation time of 19 min, others have reported generation times of 9.5-12.5 min (Schroder and Busta, 1971), 8.5 min (Bryan et al., 1971) or 24-32 min (Smith, 1963). Apparently, generation time at optimum temperature is subject to such variables as strains, growth media, age, size of inoculum, pH and redox potential among others.

An attempt was made to correlate vegetative cell numbers of *C. perfringens* with alpha toxin activity. It was found that increases in alpha toxin activity closely paralleled population increases, and that a population of at least 400,000/ml was required before alpha toxin activity could be detected by the HI test used. These data are in agreement with those found by Harmon and Kautter (1974) who showed that there is a relationship between *C. perfringens* population in
a food sample and alpha toxin activity.

Even though the results indicate that a relationship exists between the extent of growth of the organism and the amount of alpha toxin produced, several factors must be considered in interpreting the results obtained. It was observed that the incubation temperature strongly influenced the rate of alpha toxin production and the maximum yield of alpha toxin. The rate of alpha toxin production was greater at 35 than 25 C. The maximum yield of alpha toxin at 35 C was 40 times greater than at 45 C, although the growth rate of 35 C was practically the same as that of 45 C.

Optimum temperature of C. perfringens on the basis of minimum generation time was found to be 35 to 45 C. Rey, et al. (1975) observed the optimum growth temperature for this organism as being between 30 and 40 C; Breed, et al. (1957), 35 and 37 C; Collee, et al. (1961) and Smith and Holdeman (1968), 43 and 45 C.

Another factor was stability of the alpha toxin and this apparently was dependent on the temperature and time of incubation. Among the incubation temperatures examined, alpha toxin was most unstable at 35 C. At all incubation temperatures, the longer the incubation time after the stationary phase of the growth curve, the more alpha toxin was destroyed. Thus, the decline of alpha toxin activity occurred at the end of the exponential growth phase. Shemanova, et al. (1970)
demonstrated that the rapid decline of phospholipase C activity in the culture supernatant coincided with the appearance of maximum proteolytic activity. However, Nord, et al. (1974) detected little proteolytic activity and doubted that proteolytic degradation was responsible for the rapid loss of enzyme activity. This discrepancy still needs to be resolved.

The growth, alpha toxin production, and maximum yield of alpha toxin were all influenced by the nature of substrate. Growth was slower, rate of alpha toxin production was slower and yield of alpha toxin was lower when *C. perfringens* was grown in beef broth with ground beef than in Thio-glycollate medium. Thus, the amount of alpha toxin produced in any food sample may be influenced by the nature of the substrate as well as the time and temperature at which it is being held. However, Harmon and Kautter (1976) have maintained that the type of food associated with the different *C. perfringens* food poisoning outbreaks appeared to have little effect on population estimates based on the quantification of alpha toxin.

The number of organisms developing in a food system is important because it is generally accepted that approximately 100,000-1,000,000 *C. perfringens*/ml are required for an infective dose. Others (Hobbs, 1965; Hauschild and Hatcher, 1967) suggest the total dose to be 100 million organisms.
The increased use of soy protein extenders for meat products prompted the study of the effect of soy protein isolates on the growth and alpha toxin production of *C. perfringens*. When 6% Promine-D, a soy protein isolate, was blended with 14% ground beef in beef broth, it caused decreases in viable counts of *C. perfringens* instead of stimulation of the growth. Beef broth blended with 20% ground beef was employed as a control. This supported the growth and alpha toxin production of *C. perfringens*. It is of interest to note that Busta and Schroder (1971), using Trypticase (BBL) in Thioglycollate medium as a protein control, demonstrated that some soy proteins had stimulative effects on the growth of *C. perfringens*, whereas some soy proteins were inhibitory. They postulated that a modification in protein availability through manufacturing processes of soy protein and the presence or absence of some inhibitory factors might play a part in its effects on growth. Schroder and Busta (1971) also have reported that under actual meat loaf conditions, the addition of soy protein to beef did not affect the growth of *C. perfringens*.

Another area of practical significance is the wide variability between strains in alpha toxin production demonstrated by many researchers (Roberts, 1957; Nord, *et al.*, 1974; Harmon and Kautter, 1970). Harmon and Kautter (1970) have
shown that 10 of 34 strains of the *C. perfringens* isolated from suspected foods produced less than average amounts of alpha toxin and 2 of the 34 strains tested were poor alpha toxin producers. Therefore, still another precaution must be considered when relating alpha toxin activity to the actual number of the organisms or to population levels in a food product prior to processing.

Another aspect of practical significance investigated was heat inactivation characteristics of *C. perfringens* alpha toxin in the range of 55 to 95 °C in physiological saline and Thioglycollate medium. The inactivation rate of alpha toxin in both systems was linear with heating time on a semilogarithmic scale, i.e. the rate of loss of activity of alpha toxin closely obeyed first order kinetics in the range of 55 to 95 °C for 18 min.

At all the temperatures examined, greater heat inactivation was found when alpha toxin was heated in physiological saline than in Thioglycollate medium, an indication that Thioglycollate medium afforded a great degree of thermal protection to the alpha toxin. For example, after heat treatment of 18 min at 95 °C, the activity of alpha toxin remaining was 0.18% in saline and 53% in Thioglycollate medium. Thus, residual alpha toxin activity remained high when it was heated in Thioglycollate medium and could easily be detected by
the analytical procedure used. Therefore, if heat inactivation characteristics of alpha toxin in a food product are similar to those in Thioglycollate medium, any alpha toxin found would be a strong evidence that the product at some time during processing or handling had been heavily contaminated with *C. perfringens* organisms. This finding would be of major public health significance.

The nature of the thermal-protective agent (Component) of Thioglycollate medium was determined. For example, when the individual commercially available components of the Thioglycollate medium were examined as heating menstrua for alpha toxin, proteose peptone afforded alpha toxin maximum heat protection. Fractions obtained by molecular sieving of Thioglycollate medium were also tested as heating menstrua of alpha toxin. It was noted that alpha toxin was most stable in the fraction with a MW 30,000.

Therefore, it is postulated that a molecular species greater than 30,000 present in proteose peptone affords thermal protection for the alpha toxin. The exact nature of this agent and the mechanism(s) by which it protects the alpha toxin against heat destruction remain to be seen. However, it has been known for sometime that proteins may increase the stability of enzymes and other proteins. It is a common practice to use bovine serum albumin as a protective
agent for enzymes. Although the exact nature of protein protection of enzymes is unknown, a complex may take place between protein molecules or between proteins and amino acids which increases protein stability through electrostatic attraction.

Electrophoretic studies of commercial preparations of alpha toxin which may be used as standards for assay procedures revealed that the Wellcome alpha toxin preparation contained at least 7 distinct protein bands, whereas the Calbiochem alpha toxin preparation had at least 4 distinct protein bands. Practically all the alpha toxin activity was retained in a protein band for Wellcome's preparation, and in two protein bands for Calbiochem's preparation. The fact that these commercial preparations of \textit{C. perfringens} phospholipase C were not pure can be a significant one because during the last several years they have been used as important tools for a great variety of biological studies on cytotoxicity and membrane structures (Blecher, 1965; Lenard and Singer, 1968; McIllwain and Rapport, 1971; Rosenthal and Pain, 1971; Singer, 1971). The purity of the commercial products of phospholipases used was not reported in these studies, and it was not until recently that the first phospholipase C was obtained in a highly purified state (Zwaal, \textit{et al.}, 1971). Two bacterial phospholipase Cs, one from \textit{B. cereus} and one
from *C. perfringens*, have been used in these studies. However, it is well known that both these bacterial species produce many extracellular proteins such as proteases, glucosidases, neuraminidase, and theta hemolysin (Bonventre and Johnson, 1970; Bernheimer, 1970). If some of these biologically active substances were contaminated in the commercial preparations of phospholipase C, these might also alter the membrane structure. Therefore, it would be of great importance that the purity of the phospholipase C is examined before such studies are started.

In summary, the alpha toxin procedure as a means of ascertaining present or prior levels of *C. perfringens* in a food system has merit but requires further study particularly in conventional and fabricated food systems.
SUMMARY AND CONCLUSIONS

The major objective of the study was to ascertain whether or not alpha toxin can be used for the estimation of population levels of Clostridium perfringens. To achieve this objective it was necessary, (a) to evaluate various quantification procedures for alpha toxin and factors affecting their sensitivity, (b) to correlate the growth of C. perfringens and alpha toxin production under laboratory conditions, and (c) to study selected chemical and physical properties of alpha toxin.

Four procedures were evaluated for quantification of alpha toxin; (a) electroimmunodiffusion (EID), (b) radial immunodiffusion (RID), (c) lecithovitellin agar (LV) test, and (d) hemolysin indicator (HI) plate test. The first two are immunological procedures and the others are chemical tests.

Factors affecting the sensitivity of the HI test were evaluated. These included; (a) nature of diluting agents, (b) types of agar used, (c) minerals, (d) chelating agents, (e) red blood cell concentration, and (f) incubation temperature and time.

Comparison was made of the hemolytic activity of phospholipase C from C. perfringens and Bacillus cereus.

169
Growth, alpha toxin production, and pH development of *C. perfringens* were studied in: (a) Brewer Thioglycollate medium, (b) beef broth blended with 20% ground beef, and (c) beef broth blended with 14% ground beef and 6% Promine-D. In all instances, a 0.25% inoculum of overnight broth culture was used. The viable count was made by pour plates prepared with SFP base agar. Alpha toxin was quantified by the HI test.

Two commercial preparations (Wellcome Reagents Ltd., Beckenham, England and Calbiochem, San Diego, California) of alpha toxin were resolved by polyacrylamide gel (PAG) electrophoresis. The fractions obtained from PAG-SDS electrophoresis were characterized by densitometer scanning, protein determination, phospholipase C assay and hemolytic activity quantification. Hemolytic activity was quantified on HI plates made with both human and horse rbc.

The thermal inactivation characteristics of alpha toxin in saline and a Thioglycollate medium were studied at 55 to 95°C. Individual components used in the commercial preparation of Thioglycollate medium and the fractions of Thioglycollate medium obtained by molecular sieving were also utilized as heating menstrua for alpha toxin.

The results of the study would support the following conclusions:
1. Of the assay procedures successfully evaluated, the HI test was the most sensitive quantification procedure for alpha toxin.

2. For all the procedures, a linear relationship was obtained between log of the alpha toxin concentration and the reaction zone diameter.

3. The sensitivity of the HI test was 67 times greater when alpha toxin was dissolved in Thioglycollate medium than in physiological saline. The proteose peptone component of Thioglycollate medium was the major contributor to the increased sensitivity.

4. Among the types of agar examined, i.e. Epiagar, Panagar and Agar Agar No. 3, Agar Agar No. 3 resulted in the largest hemolytic zone and Epiagar, the smallest.

5. Addition of 0.3% CaCl₂ in the Epi-HI agar sharply increased the sensitivity of the HI test indicating that calcium levels in the agar are important.

6. Addition of 0.01 M EDTA or 0.3% sodium citrate in Agar Agar No. 3-HI agar completely inhibited formation of a hemolytic zone, an additional evidence for the role of calcium in the hemolytic reaction on HI plates.

7. The higher the rbc concentration in the HI plate, the smaller the hemolytic zone diameter, and vice versa.

8. The longer the incubation time, the larger the he-
molytic zone formed.

9. For the same unit, the phospholipase C of \textit{C. perfringens} produced a much greater hemolytic zone than did that of \textit{B. cereus}.

10. The optimum temperature for both growth and alpha toxin production of \textit{C. perfringens} was 35°C.

11. The rate of alpha toxin production was closely related to the growth curve. However, the maximum yield of alpha toxin did not correlate well with the growth rate.

12. The stability of alpha toxin was greatly dependent upon the temperature of incubation. Alpha toxin was least stable at 35°C.

13. In general, pH of the medium dropped when maximum growth and alpha toxin production was attained.

14. \textit{Clostridium perfringens} grew slower and produced less alpha toxin in beef broth with 20% ground beef than in Thioglycollate medium.

15. \textit{Clostridium perfringens} failed to grow in beef broth blended with 14% ground beef and 6% Promine-D.

16. Polyacrylamide gel electrophoresis revealed that the Wellcome alpha toxin contained at least 7 distinct protein bands. At least 4 distinct protein bands were observed with Calbiochem alpha toxin.

17. A major portion of the alpha toxin was present in
the protein band with an $R_m$ of 0.42 for Wellcome's preparation, and in two protein bands ($R_m$, 0.31 and 0.42) for Calbiochem's preparation.

18. Alpha toxin was much more heat resistant in Thioglycollate medium than in physiological saline. The "D" value of alpha toxin at 95°C was 6.7 min when heated in saline and 75 min when heated in Thioglycollate medium.

19. Alpha toxin was most heat stable in the proteose peptone fraction of Thioglycollate medium.

20. It is postulated that a molecular species greater than 30,000 present in proteose peptone protects the alpha toxin against heat inactivation.

21. A relationship was observed between the population level of *C. perfringens* and the amount of alpha toxin produced. In the study, the following factors were found to influence this relationship: (a) incubation time and temperature of the organism, (b) storage time and temperature of the alpha toxin, (c) nature of substrate for both, and (d) heat stability of alpha toxin. However, additional studies are needed before the alpha toxin procedure as a measure of *C. perfringens* can be utilized routinely.
REFERENCES


42. Harmon, S. M., and D. A. Kautter. 1976. Estimating population levels of Clostridium perfringens in


growth requirements for Clostridium perfringens
and isolation of auxotrophic mutants. Appl.

quantitative, qualitative, and confirmatory media
for rapid analysis of food for Clostridium

1959. Agar diffusion procedures for the assay of
lecithinase from Clostridium perfringens.

seen. Cited in "Lipolytic Enzymes" (ed. by H.
Press, New York.

111. Shemanova, G. F., V. I. Gorshkova, O. K. Borisova,
and K. L. Shakhanina. 1970. Study of the
reciprocal relationship of the exo-antigens of
Clostridium perfringens type A in the culture
medium and the bacterial cells during cultivation.
Folia Microbiologica(Praha), 15:23. Original not
Microbiol., 84:117.

112. Singer, S. J. 1971. The molecular organization of
biological membranes in "Structure and Function
of Biological Membranes" (ed. by Rothfiel, L. J.)

tion of selected whey proteins of mastitic and
normal milk by immunodiffusion. J. Dairy Sci.,
57:600.

114. Smith, L. DS., and V. M. Gardner. 1950a. The
inhibition of the lecithinase of Clostridium
perfringens by some reducing agents. J.
Franklin Inst., 250:465.

115. Smith, L. DS., and M. V. Gardner. 1950b. The
anomalous heat inactivation of Clostridium
perfringens lecithinase. Arch. of Biochem.,
25:54.


