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FACTORS AFFECTING PHYSICAL AND CHEMICAL
PROPERTIES OF ALPHA TOXIN FROM CLOSTRIDIUM
PERFRINGENS.

THE OHIO STATE UNIVERSITY, PH.D., 1976

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MARIA GREVENIOTI BABAJIMOPoulos

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FACTORS AFFECTING PHYSICAL AND CHEMICAL PROPERTIES OF
ALPHA TOXIN FROM 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

Maria Grevenioti Babajimopoulos, B.S., M.S.

* * * * * *

The Ohio State University

1978

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This dissertation is dedicated to my husband, Christos, whose patience, encouragement and sacrifices were invaluable and helped me complete this study.
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INTRODUCTION

Alpha toxin, (E.C. 3.1.4.3, phosphoglyceride diglyceride hydrolase) is a toxicogenic enzyme elaborated by Clostridium perfringens. As an enzyme, it hydrolyzes lecithin to yield phosphorylcholine and a diglyceride. It also possesses other biological functions including lethal, hemolytic, and dermonecrotic activities.

Its enzymatic nature has stimulated scientific interest particularly in the biochemistry of the reactions catalyzed and of the products formed. In contrast, toxicogenic properties of alpha toxin have been studied less extensively.

Although it has been demonstrated conclusively that alpha toxin is not the biological agent in foodborne illnesses associated with C. perfringens, alpha toxin is produced by strains of all serological types of C. perfringens but it seems to be produced in greatest amount by Type A strains. Type A C. perfringens is the third most commonly reported cause of food poisoning in man. Clostridium perfringens food poisoning results from the ingestion of a large number of vegetative cells rather than of a preformed toxin. Therefore enumeration of cell numbers is an important step for the investigation of foodborne outbreaks associated with C. perfringens. Unfortunately, enumeration of C. perfringens requires special anaerobic techniques and selective media. The organism has a low survival rate even under the most ideal
conditions, further reducing the reliability of conventional enumeration procedures.

Although alpha toxin is not involved in food poisoning by C. perfringens, some workers have suggested the possibility that the level of alpha toxin present in a food system could be used as an indirect measure of the numbers of C. perfringens organisms.

Harmon and Kautter (1970) have proposed a method in which the concentration of alpha toxin present can be utilized to estimate the extent of present and/or previous growth of C. perfringens. However, before such a procedure can be used, properties of the toxin must be understood more fully.

The purpose of the study is to increase our knowledge of the physical, chemical and biochemical properties of C. perfringens alpha toxin.
OBJECTIVES

(1) To separate phospholipase C from a commercial preparation of *Clostridium perfringens* alpha toxin;

(2) To determine hydrolytic and hemolytic activities of phospholipase C;

(3) To ascertain the effect of heat treatment on the thermal stability of alpha toxin in different menstrua; and

(4) To define the nature of the thermal protective properties of proteose-peptone for phospholipase C.
REVIEW OF LITERATURE

Type A Clostridium perfringens toxin

Serological Type A C. perfringens toxin, which produces gas gangrene in man, has been under study for almost 50 years and discoveries about its properties have been gradual.

The introduction of modern methods into protein biochemistry has permitted the isolation of separate components, the demonstration of the relation of structure to biological functions, and pharmacological, anatomical, and micropathological effects.

According to Bernheimer (1948), C. perfringens Type A toxin is a rapid acting protein of moderate potency. Van Haynigen (1950) considers its toxic activity markedly lower than the activity of tetanus and diphtheria toxins and the various types of botulinus toxins. The toxin is characterized by the presence of 12 antigens, 5 of which appear to be enzymes (Table 1).

Toxin Purification

The difficulties involved in the purification of Type A C. perfringens toxin are due to the necessity of extracting from a complex protein mixture very small amounts of a specific protein that is sensitive to variations in pH and temperature, to the nature and amount of salts present and to changes in the concentration of the associated proteins.
Table 1. Components of Type A *C. perfringens* toxin.

<table>
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<tr>
<th>Component</th>
<th>Biological Activity</th>
<th>Biochemical Nature</th>
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<td>Alpha α</td>
<td>Hemolytic, histotoxic, lethal</td>
<td>Lecithinase</td>
</tr>
<tr>
<td>Beta β</td>
<td>Histotoxic, lethal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Gamma γ</td>
<td>Lethal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Delta δ</td>
<td>Hemolytic, lethal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Epsilon ε</td>
<td>Histotoxic, lethal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Eta η</td>
<td>Lethal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Theta θ</td>
<td>Hemolytic, histotoxic, lethal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Iota ι</td>
<td>Histotoxic, lethal</td>
<td>Unknown</td>
</tr>
<tr>
<td>Kappa κ</td>
<td>Histotoxic, lethal</td>
<td>Collagenase</td>
</tr>
<tr>
<td>Lamda λ</td>
<td>Histotoxic, lethal</td>
<td>Hyaluronidase</td>
</tr>
<tr>
<td>Mu μ</td>
<td>Lethal</td>
<td>Hyaluronidase</td>
</tr>
<tr>
<td>Nu ν</td>
<td>Lethal</td>
<td>DNase</td>
</tr>
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[Source: M. V. Ispolatovskaya (1971) from data of MacLennan (1962).]
Purified *C. perfringens* toxins were first reported by Henry and Lacey (1920). The methods used were salt fractionation and precipitation by organic solvents.

Roth and Pillemer (1953) attempted purification of *C. perfringens* toxins using acid precipitation combined with fractionation with organic solvents. They achieved a 130-fold purification and the first chemical separation of the \( \alpha \) and \( \beta \) hemolysins. The final preparation indicated that the \( \alpha \)-toxin was responsible for the lethal activity of the whole toxic complex.

Haberman (1959) achieved the separation of *C. perfringens* toxin into lecithinase, hyaluronidase, and \( \alpha \)-hemolysin using a combination of precipitation by organic solvents, electrophoresis, and chromatography on hydroxyapatite. He concluded that \( \alpha \)-toxin and lecithinase were associated with the same protein molecule based on the simultaneous decrease in general toxicity and in lecithinase and hemolytic activities when the toxin was stored in the cold.

Stephen (1961) used ultrafiltration and electrophoresis on ethanol cellulose. Immunoelectrophoresis of the native toxin revealed the presence of nine antigenic components. The purified toxin, however, contained only one immunological component with low lecithinase activity.

During recent years, considerable research has been done in the Soviet Union on purification of the toxins of *C. perfringens*. Ispolatovskaya and Levdikova (1950) used starch electrophoresis and chromatography on ion exchange with a cellulose base for the separation of the collagenase, hyaluronidase and lecithinase of *C. perfringens*. The
separation was based on the different adsorption properties of these enzymes on DEAE and ECTEOLA cellulose. All of these exhibited cross contamination and required further purification. Lecithinase could be purified further by precipitation with ammonium sulfate and organic solvents and by chromatography on DEAE and carboxymethylcellulose. These steps increased the specific activity of the lecithinase. The immunoelectrophoretic pattern, which initially exhibited 9-12 zones for the native toxin, had only 2-3 zones upon purification.

Alpha toxin (Lecithinase C)

Alpha toxin (E.C. 3.1.4.3.), phosphoglyceride diglyceride-hydrolase, lecithinase C, or phospholipase C belongs to the group of hydrolases of phosphodiesters and hydrolyzes lecithin with the production of phosphorylcholine and a diglyceride as shown below.

\[
\begin{align*}
&\text{diglyceride + phosphorylcholine} \\
&\text{H}_2\text{C}-\text{O-}+\text{R}_1 \\
&\text{H} \text{C-} \text{O-}+\text{R}_2 \\
&\text{H}_2\text{C}-\text{O-P-OC}_2\text{H}_2\text{N}^+(\text{CH}_3)_3
\end{align*}
\]

In addition to being found in \textit{C. perfringens}, lecithinase has also been found in \textit{Bacillus cereus}, \textit{Clostridium oedematiens} (Macfarlane, 1941) \textit{Clostridium soldellii} (Miles and Miles, 1941) and \textit{Clostridium hemolyticum} (Macfarlane, 1948). Macfarlane has shown that the lecithinase of \textit{C.}
oedematiens of A and B types and the lecithinase of C. hemolyticum are immunologically different from that of C. perfringens.

Fox and Marshall (1976) have identified some phospholipase C producing bacteria in fresh and spoiled homogenized milk. These were Acinetobacter calcoaceticum, Alcaligenes sp., Citrobacter intermedius, Enterobacter sp., Flavobacterium devorans, Pseudomonas cichorii, Pseudomonas fluorescens, and an unidentified yeast. Later, Chrisope and Marshall (1977) found that in a model emulsion system lipolysis was greater in the presence of phospholipase C than in its absence. Phospholipase C also enhanced the activity of milk lipase in raw milk. The enhancement was much greater in milk than in the model emulsion system.

Specificity of Alpha Toxin

Macfarlane (1941) proposed two mechanisms by which bacterial toxins affect host cells. The toxin may block a metabolic reaction by competing with the substrate for the enzyme catalyzing this reaction or the toxin may exert its toxic function by attacking, according to its degree of enzyme specificity, one or more substances which are minimal constituents of a cell. It consequently distorts the metabolism of the cell either primarily by destruction of an essential structure or secondarily by the production from a normal cell constituent of a toxin substance with such powers. Thus, the toxin can affect only those cells which contain the appropriate substrate and then only if this substrate is accessible to the toxin.

Several investigators have studied the substrate specificity of lecithinase (Macfarlane, 1940, Matsumoto, 1961, Bangham and Dawson, 1962).
Macfarlane (1940) showed that in addition to lecithin the lecithinase also hydrolyzed spingomyelin but not cephalin. Matsumoto (1961) detected an essential difference in the constituents of the stroma of human and animal erythrocytes. Sheep and bovine erythrocytes are poor in lecithin and spingomyelin was the only choline-containing phospholipid present. In contrast, the erythrocytes of the horse, the rabbit, and man are rich in lecithin but poor in spingomyelin. The observed difference in the lipid constituents could be the reason for different rates and intensities of hemolysis of the erythrocytes from different animal species by the lecithinase of _C. perfringens_.

Macfarlane (1950) studied the correlation between percentage hydrolysis and percentage hemolysis of _C. welchii_ alpha toxin. She found that after an appreciable hydrolysis had occurred with little accompanying hemolysis, there was a sudden increase in hemolysis so that the latter reached 100% before the maximum hydrolysis (about 50%) took place. She concluded that the rate from which hemoglobin leaks from the cell at a particular time is a function, not directly of the rate of action of the lecithinase, but of the damage incurred by the cell at that time, i.e. the rate of leakage is constantly increasing while the rate of enzymatic action is tending to decrease as the substrate disappears.

Lecithinase C plays a fundamental role in the pathogenesis of gas gangrene. In man and most animals, the cell membrane consists of lipoprotein complexes containing lecithin. The mechanism of action of lecithinase involves the destruction of erythrocytes and leucocytes leading to a change in their permeability and in the production of edema. The edema then produces a reduction in the blood supply as well as a
reduction in the oxidation-reduction potential of the tissues. This results in an activation of the endogenous proteolytic enzymes and a resultant autolysis of the infected tissue (Elder and Miles, 1957).

Bangham and Dawson (1962) proposed that since lecithinase is a negatively charged enzyme it might attack only the micelles of phospholipids if its molecules were associated with divalent cations rendering the micelle a positive charge. Their experiments showed that calcium, magnesium and uranyl salts activated the hydrolysis of the lecithin emulsion and at the same time caused the micelles to become positively charged. Conversely, ferricyanide ions were inhibitors of the enzyme and simultaneously reversed the charge of the lipid micelles from positive to negative.

Kurioka and Matsuda (1976) studied the effect of EDTA on the activity of phospholipase C using a spectrophotometric assay method. They found that phospholipase C activity was inhibited by EDTA, but the inhibition was reversed by addition of Zn$^{++}$ and to a lesser extent by addition of Mn$^{++}$ and Co$^{++}$. This suggests that phospholipase C of _C. perfringens_ is a metalloenzyme requiring possibly Zn$^{++}$. It is of interest to note that phospholipase C of _B. cereus_ is a Zn$^{++}$ requiring enzyme.

According to Macfarlane and Knight (1941), the rate of enzymatic hydrolysis depends on the concentration of lecithin. The addition of a large amount of substrate at the time when the reaction has virtually ceased renewed hydrolysis at a rapid rate.

**Physical-chemical Properties**

The effect of various chemical compounds on the enzyme has been investigated. The enzyme was found to be resistant to thiol poisons
(p-chloromercuribenzoate and monooiodoacetate), oxidizing effect of hydrogen peroxide, and low concentrations of iodine.

Ispolatovskaya and Klimacheva (1966) have found that all the purified preparations of lecithinase contained sulfur, apparently in the form of cystine, which was detected by chromatographic analysis. The presence of cystine in the lecithinase molecule may explain its compactness and stability upon storage. However, the high reactivity of disulfide bonds may be the reason for the structural changes in the enzyme upon exposure to potassium ferricyanide or thioglycollic acid.

Jayko and Lichstein (1960) studied the effect of the presence of certain alcohols in the growth medium. At a concentration of 20% both methanol and ethanol caused greater than 90% inhibition of lecithinase activity with a concomitant 50% reduction in growth. The inhibitory effect of alcohols increased with increasing molecular weight. When alcohols were added individually to aliquots of culture supernatant, they had no effect on stability of the preformed toxin suggesting that their effect during growth was on inhibition of toxin synthesis.

Although lecithinase of \textit{C. perfringens} is an exoenzyme, lecithinase activity was demonstrated in cytoplasmic extracts obtained from mechanically disrupted vegetative cells of Type A \textit{C. perfringens} (Meisel et al., 1959). Studies by Nakamura and Cross (1958) have indicated that strains of \textit{C. perfringens} that produce large quantities of lecithinase excrete most of their lecithinase into the surrounding medium whereas, strains of \textit{C. perfringens} that produce low lecithinase activity retain approximately 50% of the enzyme in the cytoplasm. Therefore, the total lecithinase activity of \textit{C. perfringens} may not be determined if only culture filtrates are assayed for lecithinase activity.
Several investigators (Jayko and Lichstein, 1959; Nakamura and Cross, 1968; Nakamura et al., 1968) have studied factors that influence lecithinase production by *C. perfringens*. They found that the presence of an adequate supply of peptides stimulates alpha toxin production. Murata et al. (1965) have demonstrated that arginine was required by *C. perfringens* for the production of alpha toxin.

Smith and Gardner (1950) conducted studies of the heat inactivation of alpha toxin. Crude lecithinase was heated at 50, 60, 70, 80, 90, and 100°C for 10 min. Their data indicated that heating at temperatures in excess of 60-70°C inactivated less enzyme than heating at lower temperatures. In addition, the greater the dilution of the enzyme the less the inactivation.

It was thought that the enzyme might be associating to form an inactive complex probably bound through some divalent ions, e.g. calcium and magnesium, which may be present in the casein digest culture medium and in crude lecithinase preparations. The "anomalous" heat inactivation was almost entirely abolished by freeing the enzyme of easily dissociable ions by means of electrodialysis. The phenomenon could be restored by the addition of calcium or magnesium.

Smith and Gardner (1950) also found that the enzyme activity of heated toxin (65°C for 10 min.) was increased on being heated again at 100°C for 10 min. They concluded that complex formation did take place at 65°C and that the complex was dissociated at 100°C with the consequent release of the enzyme.

Park (1976) investigated the heat inactivation characteristics of *C. perfringens* alpha toxin in the range of 55-95°C in physiological
saline and Thioglycollate medium. A striking feature was that the "D" values were much greater at all temperatures studied when the toxin was heated in Thioglycollate medium than in saline. In addition, when the alpha toxin was heated in Thioglycollate medium, the thermal inactivation time curve exhibited a very unusual shape. The curve failed to show a definite negative slope suggesting an unusual heat resistance.

A similar phenomenon was observed by Jamlang et al. (1971) with heat inactivation studies of staphylococcal enterotoxin B. When staphylococcal toxin solutions inactivated at 70°C and 80°C were reheated at 100°C for 6 min., a recovery of 35-40% of the initial activity was obtained. Their explanation was that heat aggregation at 70°C and 80°C could be reversed by higher temperatures due to the low bond energy of the coagulation sites.

Hofstee (1949) has shown that as much as a five-fold increase in urease activity can be obtained when a sample previously inactivated by storage for 24 hr at -10°C is heated for 5 min. at 60°C. His data suggest that an association between urease molecules or between urease and other proteins might occur resulting in inactivation of the enzyme which could be reversed on dissociation.

Measurement of Activity on Selected Substrates

Macfarlane et al. (1941) confirmed that C. welchii alpha toxin when mixed with some human sera produces a marked opalescence within a few hours. When examined under the dark field, the milky material is seen to contain large numbers of refractile particles resembling those of fat. They advanced two possible explanations: a) the fat previously so finely divided as to be invisible had been aggregated into a visible
form by the toxin, and/or b) the fat originally in combination with other material such as protein was set free.

In order to test these postulations, the effect of the toxin on a known lipoprotein was investigated. Crude lecithovitellin was prepared by mixing the yolk of an egg in saline and filtering the mixture through a Seitz filter. When this crude lecithovitellin mixture was treated with \textit{C. welchii} toxin, opalescence appeared within a few minutes, proceeding to flocculation and then separation of a thick curd of fat. When lecithovitellin was extracted with ether and then treated with ether-extracted \textit{C. welchii} toxin, a much reduced opalescence occurred, however, more fat could be extracted from the opalescent liquid. They concluded that the greater part of the opalescence produced was due to the aggregation of finely divided free fat and a small part resulted from freeing of the fat from compounds, probably lipoproteins, since some protein was denatured at the same time.

Zamecnik \textit{et al.} (1947) thought that the course of the enzymatic reaction of lecithinase (\textit{\alpha}-toxin) might be followed manometrically based on the following theoretical reasoning: if phosphorylcholine (pk 5.6) is liberated from lecithin by \textit{C. welchii} lecithinase, it should drive off carbon dioxide from the reaction mixture buffered in the region of pH 7 by a bicarbonate-carbon dioxide buffer system. A method based on this principle has been worked out. The activity of the enzyme was measured in a Warburg manometer. The reaction mixture consisted of lecithin, sodium bicarbonate, calcium chloride and enzyme diluted in 1% albumin for stability.

Another method used by Macfarlane and Knight (1941) was based on colorimetric measurement of inorganic phosphorus. A unit was defined
as that activity causing the liberation of one micromole of inorganic phosphate per min. at 37°C.

Zwaal et al. (1971) measured phospholipase C activity by a modified titrimetric method. The release of titratable H\(^+\) was followed in a recording titration unit during continuous addition of 0.01 M NaOH. The reaction mixture was kept at 37°C and pH 7.2. The mixture was continuously agitated with a teflon-coated magnetic stirrer and kept under a nitrogen atmosphere. One unit of phospholipase C was defined as the amount of enzyme which liberated one micromole of titratable H\(^+\) per min.

This method was accurate, but was time-consuming for routine work or for assay of large number of samples. Phosphorylcholine is very stable to acid hydrolyzing reagents and thus requires treatment by wet ashing in sulfuric acid. Kurioka and Liu (1966) reported an improved assay method for phospholipase C, combining the use of an anionic detergent and alkaline phosphatase. Alkaline phosphatase is used for quantitative liberation of inorganic phosphate directly from the phospholipase C reaction mixture without acid digestion.

Kurioka and Matsuda (1976) introduced a spectrophotometric method for the assay of phospholipase C. It was based on the fact that phospholipase C hydrolyzes p-nitrophenylphosphorylcholine (NPPC) to phosphorylcholine and p-nitrophenol, a chromogen. The hydrolytic rate of NPPC by phospholipase C in aqueous medium was, however, too low to measure the enzyme activity. The method was improved by the addition of sorbitol or glycerol which increased the hydrolytic rate considerably. They suggested that the presence of sorbitol or glycerol may improve the environment of the reaction medium. A hydrophobic environment surrounding NPPC
and phospholipase C was suspected to exist as a result of hydration of the sorbitol or glycerol added to the reaction medium.

Two methods are referred to the "Compendium of Methods for the Microbiological Examination of Foods" (1976): a) the hemolysin indicator plate method and b) the lecithovitellin test. The first is based on the formation of a clear zone on a blood-agar plate upon hemolysis of human red blood cells. The zone is proportional to the enzyme activity. The second is the formation of a white pellicle on a lecithovitellin-lecithinase mixture.

Park and Mikolajcik (1977) studied the application of electro-immunodiffusion on cellulose acetate, radial immunodiffusion, lecithovitellin agar plate (LV) and hemolysin indicator plate (HI) for the quantification of *C. perfringens* alpha toxin. Their results revealed that the hemolysin indicator plate method was the most sensitive detecting 0.0003 unit/ml. They also studied factors affecting the sensitivity of the HI plate method and found that the sensitivity of the test was greater when alpha toxin was dissolved in Thioglycollate medium than in physiological saline. Among the types of agar examined, *e.g.* Epiagar, Panagar, and Agar Agar No. 3, Agar Agar No. 3 yielded the largest hemolytic zone. Addition of EDTA or sodium citrate to Agar Agar No. 3 HI plates inhibited the formation of a hemolytic zone. This would indicate that the presence of calcium in the agar is important since the addition of Ca$^{++}$ overcame the inhibition. The size of the hemolytic zone was found to be directly proportional to the incubation time and inversely proportional to the human red blood cell concentration in the HI plates.
Harmon and Kautter (1970, 1977) used the HI plate method and the LV test to estimate previous growth of *C. perfringens* in food samples. Although their results indicate a relationship between the extent of growth of the organism and the amount of alpha toxin produced, they suggest that several factors must be considered in interpreting the results obtained by the HI method. The amount of alpha toxin produced in a food may be influenced by the nature of the substrate as well as the time and temperature at which it was held. The recovery of alpha toxin is dependent on the extraction method used. In addition some strains which produce small quantities of alpha toxin may not be detected by this method. Results obtained with food extracts indicated that the HI plate method is more sensitive than the LV test. Some extracts from canned chicken broth, however, failed to give a positive LV test while exhibiting hemolytic activity by the HI plate method.

It is apparent that our knowledge of the phospholipase C of *C. perfringens* is limited and additional basic research is needed before the HI or LV procedure can be used to assay indirectly for the presence of *C. perfringens* in food systems.
MATERIALS AND METHODS

Toxin and Antiserum

Standardized C. perfringens alpha toxin was obtained from Wellcome Reagents, Limited, Beckenham, England. Anti-alpha toxin was obtained from the same source. Potency of the preparations was furnished by the supplier.

Purification Methods

Column Chromatography

Gel filtration was used to purify the commercial preparation of the alpha toxin. Sephadex G-100 was used in a 75 x 1.8 cm column. The elution buffer was 0.05 M Tris-HCl, pH 7.6, containing 5.0 mM CaCl$_2$ and 0.1 M KCl, with a flow rate of 7.2 ml/hr. Four milliliter fractions were collected. Column chromatography was also used to study the complex formation between alpha toxin and proteose-peptone upon heat treatment. For this phase of the study, Sephadex G-75 was used in a 80 x 1.8 cm column. The flow rate was 5 ml/hr and 4 ml fractions were collected.

Eluting buffer was Tris-HCl buffer, pH 7.4.

Immunoelectrophoresis and Immunodiffusion

Basically, immunoelectrophoresis was run according to Arquemburg et al. (1970). Run time was 1.5 hr in 0.1 M barbitone acetate buffer, pH 8.6. For preparation of the gel, Oxoid ID agar tablets were used. The voltage applied was 20 mAmps per holder containing 7 microscopic slides.
Immunodiffusion was run according to Crowle (1975). Each plate was developed at room temperature in a moisture chamber for 24 hr.

For both methods, the Wellcome preparation of anti-serum was used. The plates were stained with 0.5% amido black in 6% acetic acid for about 30 min. Excess dye was removed by soaking for 2-3 hr in acetic acid and then overnight in 0.85% NaCl.

**Polyacrylamide Gel Electrophoresis**

The technique used was a modification of the method used by Park (1976) based on the method described by Laemmi (1970). The gel consisted of two parts: separating gel (lower gel) and stacking gel (upper gel). The separating gel (10% polyacrylamide) was prepared by mixing 18 ml of acrylamide stock solution (22.2 g acrylamide + 0.6 g methylene bisacrylamide in a final volume to 100 ml), 10 ml of 1.5 M Tris-HCl buffer containing 0.4% sodium dodecyl sulfate (SDS, pH 8.8), 1 ml of 0.1% ammonium persulfate, 11 ml of water, and 0.03 ml TEMED. After polymerization of the separating gels (6 cm in length), the stacking gels (7 mm in length) were formed. 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 0.1% ammonium persulfate, 10 ml of water, and 0.01 ml TEMED. After polymerization of the upper gels, 50 μl of the sample and 10 μl of 0.1% bromophenol blue were applied on the top of the gels. Electrophoresis was run with a current of 2 mA/tube in a pH 8.3 buffer containing 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. After electrophoresis, the gels were stained overnight with 0.4% coomassie brilliant blue with 50% methanol and 9.2% acetic acid. They were then destained overnight with 5% methanol and 9.2% acetic acid.
The protein content of the alpha toxin and the various fractions collected from the gel chromatography was determined by the Lowry et al. method (1951). A standard curve was prepared with crystalline bovine serum albumin.

**Phospholipase C Activity**

**Lecithovitellin Agar Method**

To prepare the lecithovitellin solution, egg yold was suspended in physiological saline (20% v/v), centrifuged at 15,000 RCF for 15 min., and the supernatant was sterilized by passage through a Seitz filter (0.45 μ). Agar Agar No. 3 (Oxoid) at a concentration of 1.7% was prepared in physiological saline. The agar (90 ml) was cooled to 45°C and 10 ml of the lecithovitellin solution were added. This was dispersed in Petri dishes which were allowed to air cool. Prior to use, test wells (3 mm) were cut into the agar with a thin-walled metal die. Five microliter samples were then applied in the test wells. The plates were incubated at 37°C for 24 hrs. The diameter of reaction zone was measured using a microcaliper with a ± 0.1 mm precision.

**Hemolysin Indicator Plate Method**

Agar Agar No. 3 (1.7%) was prepared in physiological saline. Packed human red blood cells were obtained from the American Red Cross Blood Bank, Columbus, Ohio. The cells were washed three times with four volumes of sterile physiological saline between centrifugations at 3,000 RCF for 15 min.

Twelve milliliters of washed red blood cells were added to 100 ml of Agar Agar No. 3 at 45°C and mixed thoroughly. Ten milliliters of the above mixture were dispensed per each Petri dish. The plates were dried
overnight and stored at 4°C. Prior to use, test wells (3 mm) were cut into the blood agar. Five microliters of sample were used per well. The diameter of hemolytic zone was measured using a microcaliper with a + 0.1 mm precision.

Spectrophotometric (NPPC) Assay

The method of Kurioka and Matsuda (1976) was used with p-nitrophenyl phosphorylcholine as substrate. The reaction mixture consisted of 2.3 ml of 0.25 M Tris-HCl buffer, pH 7.2, containing 60% sorbitol (w/w) and 0.2 ml of pure alpha toxin containing 0.04 mg of protein. Total volume was 2.5 ml. The substrate concentrations were 5, 10, 20, 40 mM. The reaction was run for 30 min. at 35°C and the % transmittance was read at 410 nm using a 1 cm cuvette. The reading was converted to absorbance from a standard table. The molar extinction coefficient of p-nitrophenol was 15,400 cm⁻¹ M⁻¹.

Assay for Heat Inactivation of Alpha Toxin

For heat inactivation of alpha toxin, 0.05 ml of alpha toxin solution in the desired substrate was placed in a series of glass test tubes (75 mm length, 8 mm inside diameter, 0.2 mm wall thickness). The tubes were then placed in ethylene glycol bath equilibrated at the desired temperature.

At the desired time intervals, the test tubes were removed and placed into an ice-water bath. Five microliter portions of the heated alpha toxin solution were spotted in the test well of the HI plates. The hemolytic zones were read after 24 hrs incubation at 35°C.

The D value, which is the time required for the toxin concentration to be decreased by 90%, was calculated from the equation
\[ D = \frac{t}{\log b - \log a} \]

where \( t \) was the heating time, \( b \), the toxin concentration at initial time, and \( a \), the toxin concentration remaining after heating time \( t \).

**Treatment of Data**

For the lecithovitellin agar (LV) test and hemolysin indicator plate (HI) test, standard curves were made by plotting the log of the toxin concentration versus the diameter of the reaction zone. The best straight line was calculated by the least square method. Unknown concentrations were read from the standard curve (Figures 6-7, Appendix).

For the NPPC assay method the moles of p-nitrophenol formed were calculated using the equation

\[ A = C \cdot E \cdot \varepsilon \]

and solving for

\[ C = \frac{A}{E \cdot \varepsilon} \]

where \( A = \) absorbance, \( C = \) concentration, \( \varepsilon = \) path length, in cm and \( E = \) molar extinction coefficient in cm\(^{-1}\) M\(^{-1}\).
RESULTS

Purification of C. perfringens alpha toxin

The first phase of the study involved purification of a commercial preparation (Wellcome Reagents, LTD.) of C. perfringens alpha toxin to determine whether or not the phospholipolytic and hemolytic activities reside with the same protein molecule.

The following procedures were utilized for purification of alpha toxin: a) gel filtration, b) polyacrylamide disc gel electrophoresis (PAGE), c) immunodiffusion, and d) immunoelectrophoresis.

Primary Gel Filtration

The Wellcome preparation of alpha toxin diluted in 0.85% NaCl was placed on a 75 x 1.8 cm Sephadex G-100 column. The elution buffer was 0.05 M Tris-CH1, pH 7.6, containing 5.0 mM CaCl₂ and 0.1 M KCl with a flow rate of 7.2 ml/hr. Four milliliter fractions were collected and each fraction was assayed for hemolytic and phospholipolytic activities. Absorbance was read at 380 nm using a UV monitor. Hemolytic activity for the presence of α-hemolysin was checked by the HI plate method using horse red blood cells.

Theta hemolysin was determined because it has been reported to be one of the components of Type A C. perfringens toxin. Thus, it might be present in the commercial preparation of alpha toxin where it would contribute to the hemolytic activity. Theta hemolysin can attach both human and horse red blood cells, whereas phospholipase C attacks only
human red blood cells. Therefore, hemolytic activity against horse red blood cells would be good evidence for the presence of β-hemolysin.

Elution patterns for hemolytic activity, phospholipase C activity and absorbance at 280 nm are shown in Fig. 1. Phospholipolytic activity and most of the hemolytic activity were located in fractions (tubes) 16-22. Fractions 22-30 had some hemolytic activity but were also positive for β-hemolysin activity shown as cross marks on the figure. Major absorbance at 280 nm was located with material collected in tubes 25-35.

The contents of tubes 16-23 were combined, dialyzed against distilled water for 24 hr, and concentrated against polyethylene glycol (PEG 20). The concentrate was checked for homogeneity by double immunodiffusion, immunoelectrophoresis and SDS polyacrylamide disc gel electrophoresis. Protein content was determined by the Lowry et al. procedure (1951).

Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoresis of the commercial toxin revealed the presence of at least 7 distinct protein bands (Fig. 2A). After gel filtration, the contents from tubes 16-22 showed only 2 distinct bands at Rm 0.64 and 0.28 (Fig. 2B, 3).

A duplicate unstained gel was placed next to a stained gel in order to locate areas of interest and the unstained gel was cut into 5 fractions based upon relative mobility (Rm). The Rm of the areas making up each fraction is shown in Fig. 2. Each fraction was macerated in distilled water and held overnight. Fractions were then assayed for phospholipase C activity. Only the fraction which contained the protein
Figure 1. Separation of commercial alpha toxin on a Sephadex G-100 column. Four ml fractions were collected. A, hemolytic activity; B, phospholipase C activity; and C, absorbance at 280 nm. Cross symbols represent points at which fractions exhibited α-hemolysin activity.
Figure 1

Absorbance 280nm (C)

Hemolytic Activity [HU/ml] (A)

Phospholipase C Activity [LYU/ml] (B)
Figure 2. Disc electrophoretic histograms of A, commercial alpha toxin, B, partially purified alpha toxin, C, purified alpha toxin. Relative mobilities (Rm) of the areas cut from the gel are shown.

Rm
1. 1  - 0.806
2. 0.806 - 0.551
3. 0.551 - 0.290
4. 0.290 - 0.145
5. 0.145 - 0
Figure 3. Densitometer scan of partially purified alpha toxin (first gel filtration) upon polyacrylamide disc gel electrophoresis.
band with a Rm of 0.64 possessed both hemolytic and phospholipolytic activities whereas, the other fractions were inactive.

**Immunodiffusion and Immunoelectrophoresis**

Double immunodiffusion of the commercial toxin revealed the presence of three bands (Fig. 4). However, the partially purified toxin revealed the presence of only one band. This does not necessarily mean that there is only one antigen present. The number of precipitin bands developing in a double diffusion test may be interpreted as representing the minimum number of precipitating systems present and not necessarily the maximum number present.

Immunoelectrophoresis of the partially purified alpha toxin when developed by antiserum to the commercial preparation revealed the presence of two bands (Fig. 5B). On the other hand, the commercial preparation of alpha toxin exhibited five bands (Fig. 5A). Because SDS disc gel electrophoresis revealed seven protein bands, the results of the immunoelectrophoresis analysis would indicate that some of the antigens present do not form strong precipitation lines with their specific antibodies and/or some of the antigens exhibit overlapping electrophoretic mobility.

**Second Gel Filtration**

For further purification, the active fraction, which was obtained from the first gel filtration, was again subjected to gel filtration with a flow rate of 4 ml/hr. This time 2 ml fractions were collected. In the elution pattern shown in Fig. 6, 2 peaks were observed. The first peak corresponded to tubes 18-24 and the second to tubes 30-42. Hemolytic and phospholipolytic activities were present in the latter
Figure 4. Immunodiffusion patterns of commercial and purified alpha toxin. A, a, commercial alpha toxin. B, a, commercial alpha toxin, B, b, purified alpha toxin, and B, c, antiserum to commercial alpha toxin.
Figure 5. Immunoelectrophoretic patterns of A, commercial alpha toxin, B, partially purified alpha toxin, and C, purified alpha toxin.
Figure 6. Separation of partially purified alpha toxin on a Sephadex G-100 column. Two ml fractions were collected. A, hemolytic activity, B, phospholipase C activity, and C, absorbance at 280 nm.
Figure 6

Absorbance 280 nm (C)

Hemolytic Activity [HU/ml] (A)

Phospholipase C Activity [LVU/ml] (B)
tubes. This time, a-hemolysin activity was not observed. It appears that a-hemolysin was removed during the first gel filtration. The contents of the active tubes were combined, dialyzed overnight against distilled water, and concentrated against PEG 20 to a total volume of 2 ml.

Polyacrylamide disc gel electrophoresis with SDS revealed the presence of only one protein band with a Rm of 0.64 which possessed both hemolytic and phospholipolytic activities (Figs. 2C and 6). Treatment of the sample with mercaptoethanol and urea did not alter the electrophoretic pattern (Fig. 7), an indication that there was no protein aggregation through S-S or hydrogen bonds.

Immunelectrophoresis of the same fraction revealed the presence of one precipitin band moving towards the cathode (Fig. 5C). According to Bangham (1962), the isoelectric point of phospholipase C is 5.1-5.2 and thus one would expect that the band would move toward the anode. The phenomenon of reversed electrophoretic mobility may be due to electroendoosmosis. At times, this is a desired phenomenon since it gives better resolution when several antigens are present in a preparation.

Table 2 shows the protein content, the total and specific activities and the percent recovery of activity of the fractions obtained at different steps of purification. The percent recovery of the hemolytic activity after the first passage through the gel was 53.4% whereas, the recovery of the phospholipolytic activity was 97%. Since the enzyme had the same amount of total hemolytic and phospholipolytic activity before purification, it seems that part of the hemolytic activity was lost in the first gel filtration step. This can be attributed to the separation
Figure 7. Densitometer scan of purified alpha toxin (second gel filtration) upon polyacrylamide disc electrophoresis.
Table 2. The protein content, hemolytic activity, and phospholipolytic activity of alpha toxin purified by Sephadex G-100 filtration.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (HU)</th>
<th>Recovery (%)</th>
<th>Specific Activity (HU/Mg Protein)</th>
<th>Increase In Specific Activity (%)</th>
<th>Total Activity (LV units)</th>
<th>Recovery (%)</th>
<th>Specific Activity (LVU/mg Protein)</th>
<th>Increase In Specific Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Gel Filtration</td>
<td>2</td>
<td>19.8</td>
<td>50</td>
<td>--</td>
<td>2.5</td>
<td>--</td>
<td>50</td>
<td>--</td>
<td>2.5</td>
<td>--</td>
</tr>
<tr>
<td>Sephadex G-100 (1st Passage)</td>
<td>2.3</td>
<td>6.2</td>
<td>26.7</td>
<td>53.4</td>
<td>4.3</td>
<td>73.5</td>
<td>48.5</td>
<td>97</td>
<td>7.8</td>
<td>215.0</td>
</tr>
<tr>
<td>Sephadex G-100 (2nd Passage)</td>
<td>2.0</td>
<td>1.8</td>
<td>19.3</td>
<td>61.1</td>
<td>10.7</td>
<td>370.4</td>
<td>30.1</td>
<td>62.1</td>
<td>16.2</td>
<td>547.0</td>
</tr>
</tbody>
</table>
and removal of α-hemolysin which may have contributed to some of the hemolytic activity. After the second gel fraction step, the percent recovery of the 2 activities was close e.g. 61.1% for hemolytic activity and 62.1% for phospholipolytic activity. There was a four-fold increase in hemolytic specific activity after the final purification step and a six-fold increase in the phospholipolytic specific activity. This difference can also be explained as a loss of part of the hemolytic activity.

The reaction of alpha toxin with its homologous antiserum was made in order to determine whether antigenic sites and enzymatic sites were the same. For this experiment, equal volumes of purified alpha toxin and anti-alpha toxin serum (diluted 1:4 with saline) were mixed and permitted to react for 1 hr at room temperature. Then the hemolytic and phospholipolytic activities were assayed on HI plate and LV plate respectively. After incubation of the plates at 37°C for 24 hr, both tests were negative. Hemolytic and phospholipolytic activities were completely inhibited by the reaction of alpha toxin with its anti-toxin serum. This indicated that enzymatic sites are also antigenic sites and that both activities may have the same enzymatic site.

Heat Inactivation and Reactivation

Park (1976) has shown that a proteose-peptone fraction with a MW > 30,000 protected alpha toxin against heat inactivation. The thermal inactivation time curve was similar to that observed in Thioglycollate medium. Proteose-peptone is one of the components of Thioglycollate medium and was the only one which exhibited substantial thermal protective properties.
"D" Values in Proteose-Peptone and Saline

Using Diaflow PM50 ultrafiltration membrane with an exclusion size of 50,000, proteose-peptone was separated into 2 fractions: one with a MW ≤ 50,000 and another with a MW ≥ 50,000. Commercial and purified preparations of alpha toxin were heated in each fraction and in physiological saline (0.85% NaCl). Heating temperatures were 55, 65, 75, 85, and 95°C for 6, 9, 12, 15, and 18 min. The "D" values obtained are shown in Table 3. It is obvious that the fraction with a MW ≥ 50,000 was the most protective. The alpha toxin was not destroyed at temperatures up to 85°C. At 95°C, some destruction was observed with a "D" value of 444 min.

The log of the "D" values was plotted against the corresponding temperature to construct a thermal inactivation curve. When commercial alpha toxin was heated in NaCl and in the proteose-peptone fraction having a MW ≤ 50,000, the thermal inactivation time "Z" curve exhibited an expected negative slope between 55°C and 65 or 75°C. However, beyond these temperatures the curve exhibited an anomalous response in that greater heat resistance was evident for the alpha toxin at 85°C than at 75°C (Fig. 8). The purified alpha toxin showed a thermal inactivation curve with a negative slope when heated in the same proteose-peptone fraction and a broken curve below and above 65°C when heated in physiological saline (Fig. 9).

Complex Formation

In order to determine whether the heat protective effect was due to a complex formation between proteose-peptone and alpha toxin, it was first necessary to establish the elution patterns of each of these
Table 3. "D" values for commercial alpha toxin and purified alpha toxin in physiological saline and in proteose-peptone (PP) fractions.

<table>
<thead>
<tr>
<th>Heating Temp. (°C)</th>
<th>Commercial α-toxin Heating Menstrua</th>
<th>Purified α-toxin Heating Menstrua</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCl</td>
<td>PP&lt;50T (min)</td>
</tr>
<tr>
<td>55</td>
<td>90</td>
<td>300</td>
</tr>
<tr>
<td>65</td>
<td>26.8</td>
<td>73.1</td>
</tr>
<tr>
<td>75</td>
<td>25.8</td>
<td>43.9</td>
</tr>
<tr>
<td>85</td>
<td>28.5</td>
<td>56.9</td>
</tr>
<tr>
<td>95</td>
<td>21.8</td>
<td>47.3</td>
</tr>
</tbody>
</table>

Average of two trials.
Figure 8. Thermal inactivation time curve of commercial alpha toxin in physiological saline and in the proteose-peptone fraction with a MW < 50,000.
Figure 8

D value [min] vs. Temperature °C

- Proteose peptone
- NaCl
Figure 9. Thermal inactivation time curve of purified alpha toxin in physiological saline and in a proteose-peptone fraction with a MW $\leq$ 50,000.
Figure 9

- Proteose peptone
- \( T = 0.981 \)
- NaCl
agents on a Sephadex G-75 column. The elution buffer was Tris-HCl, pH 7.4. The proteose-peptone or alpha toxin was suspended in the elution buffer. Three milliliter fractions were collected and absorbance was read at 280 nm. For the proteose-peptone, four peaks were observed (Fig. 10). One peak came out with the void volume indicating a MW > 75,000, another with an average MW of 60,000, and 2 large peaks at an average MW of 19,000 and ~14,000.

Pure alpha toxin showed only one peak (Fig. 10). The activity of all 45 fractions collected was assayed on HI plates. The peak of hemolytic activity coincided with the peak of maximum absorbance.

In order to prepare a standard curve for the determination of the MW of the fractions, proteins of known MW i.e. bovine serum albumin (69,000), β-lactoglobulin (36,000) and α-lactalbumin (14,000) were passed through the same gel column. The void volume was determined using blue dextran. A standard curve was constructed by plotting the MW versus the ratio \( \frac{Ve}{Vo} \) where \( Ve \) was elution volume, and \( Vo \) was void volume (Fig. 11).

A mixture of alpha toxin in the proteose-peptone fraction was heated at 95°C for 18 min. It was cooled and then subjected to Sephadex G-75 gel filtration utilizing the same column. An unheated mixture was also run. Three ml fractions were again collected and assayed for alpha toxin activity by HI method. As is shown by the cross symbols on the elution pattern of the unheated mixture, activity was located in tubes 24-28 at the same volume where the pure toxin alone was eluted (Fig. 12). For the heated mixture of alpha toxin in proteose-peptone, activity was present in tubes 14, 19-22, and 27 and from the standard curve (Fig. 11), the elution volume of alpha toxin corresponded to a MW of ~45,000 (Fig. 11).
Figure 10. Elution patterns on a Sephadex G-75 column of the proteose-peptone fraction having a MW > 50,000, A, and alpha toxin, B. The cross symbols indicate hemolytic activity.
Figure 10

A Proteose Peptone

B alpha toxin

Absorbance 280 nm
Figure 11. Standard curve for Molecular weight determination on Sephadex G-75 column.
Figure 12. Gel filtration on Sephadex G-75 column of mixture of unheated alpha toxin and proteose-peptone, A, and of the same system after heating at $95^\circ$C for 18 min., B. Cross symbols indicate areas of hemolytic activity.
The fractions eluted from the heated mixture of alpha toxin and proteose-peptone had a MW of either \( \sim 75,000 \) or \( 58,000 \) (Fig. 12). Therefore, the possible complexing molecules of proteose-peptone would have MW \( \geq 30,000 \) and \( \sim 13,000 \).

In order to determine whether a specific fraction of proteose-peptone was responsible for the protective effect against heat inactivation of alpha toxin or whether a combination of two or more fractions was involved, four fractions of proteose-peptone which corresponded to the different absorbance peaks were collected. Specifically, each fraction identified in Table 4 was formed from the contents of the tubes collected (Fig. 10). The fractions were concentrated against PEG to a volume of about 2 ml. Alpha toxin was then heated in each fraction at 95°C for 18 min. Combinations of equal volumes of the fractions were also used as heating menstrua. The percent residual activity of alpha toxin and the \( D_{95} \) values are also shown in Table 4. It can be seen from combination of Fractions B and C that these were highly protective, \( D_{95} = 164 \) min. Apparently, the presence of high and low molecular weight agents is required for protection of the alpha toxin against heat inactivation.

**Nature of the Reaction Involved in Complex Formation**

In order to determine whether a disulfide interchange reaction was involved in complex formation, the effect of the reagent NEM (N-ethylmaleimide) on the heat inactivation of alpha toxin in the presence of proteose-peptone was studied. N-ethylmaleimide ties up the -SH groups so that they are no longer available for an \(-S-S-\) interchange reaction. N-ethylmaleimide was added to the alpha toxin-proteose-peptone solution at a \( 10^{-3} \) M concentration. The mixture was permitted to react
Table 4. Percent residual activity and $D_{95}$ values for purified alpha toxin heated in different fractions of proteose-peptone at 95°C for 18 min.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Tube No.</th>
<th>% Residual Activity</th>
<th>$D_{95}$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12-16</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td>B</td>
<td>17-27</td>
<td>38</td>
<td>43</td>
</tr>
<tr>
<td>C</td>
<td>30-40</td>
<td>31</td>
<td>38</td>
</tr>
<tr>
<td>D</td>
<td>41-46</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>B + C</td>
<td></td>
<td>78</td>
<td>164</td>
</tr>
<tr>
<td>A + C</td>
<td></td>
<td>54</td>
<td>73</td>
</tr>
<tr>
<td>A + B</td>
<td></td>
<td>57</td>
<td>72</td>
</tr>
<tr>
<td>A + B + C + D</td>
<td></td>
<td>83</td>
<td>200</td>
</tr>
</tbody>
</table>

(1) See Figure 10 for location of tubes in the elution pattern.

(2) Average of two trials.
at room temperature. The reaction was followed spectrophotometrically at 305 nm. The end of the reaction was the time when absorbance stopped decreasing.

Three different experimental treatments were prepared: one contained alpha toxin, proteose-peptone and NEM and was not heated. The second contained alpha toxin in proteose-peptone followed by a heat treatment at 95°C for 18 min. with the NEM added after heating. The third treatment contained the three components heated together at 95°C for 18 min.

The results obtained are shown in Table 5. N-ethylmaleimide did not have any effect on the percent residual activity of alpha toxin when added to the unheated mixture of α-toxin-proteose-peptone (Treatment One) or to the mixture after being heated at 95°C for 18 min. (Treatment Two). However, when the mixture was heated in the presence of NEM (Treatment Three), the toxin was inactivated and the protective effect was not observed. This would indicate that NEM prevents the complex formation through the disulfide interchange reaction by blocking the -SH groups.

The effect of Ellman's reagent [5, 5-dithiobis (2-nitrobenzoic acid)] on heat inactivation of alpha toxin in the presence of proteose-peptone was also investigated. Ellman's reagent reacts with -SH groups making them unavailable for -SH -S-S-exchange reaction. For this treatment, 40 mg of Ellman's reagent were dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.0. Two different samples were prepared.
Table 5. Effect of N-ethylmaleimide on heat inactivation of alpha toxin suspended in proteose-peptone.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Residual Activity</th>
<th>D_{95} Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) $\alpha$-toxin + PP + NEM</td>
<td>100 ± 0.8</td>
<td>--</td>
</tr>
<tr>
<td>unheated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) $\alpha$-toxin + PP heated</td>
<td>100 ± 0.8</td>
<td>--</td>
</tr>
<tr>
<td>at 95°C for 18 min. then add NEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) $\alpha$-toxin + PP + NEM</td>
<td>16</td>
<td>22.7</td>
</tr>
<tr>
<td>heated at 95°C for 18 min.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1) Average of two trials.
(2) + values indicate percent error based on standard curve (Fig. 18, Appendix).
Sample A (Control)  
2 ml phosphate buffer, pH 7  
0.05 ml alpha toxin  
0.2 ml of 1% proteose-peptone  
1.04 ml of water  
0.04 ml Ellman's reagent  
Sample B  
5 ml phosphate buffer, pH 7  
0.05 ml alpha toxin  
0.2 ml of 1% proteose-peptone  
1.0 ml water  

Sample A was the control system in which heat protection by proteose-peptone was observed to occur. For Sample B, the system was allowed to react at room temperature but with Ellman's reagent. The reaction was followed by reading the absorbance at 410 nm. The reaction was ended when no further increase in absorbance occurred. At this point, 0.05 ml aliquots from both samples were taken. These were heated in glass tubes at 95°C for 0, 6, 12, and 18 min. Hemolytic activity was assayed by the HI method. The percent residual activity and the $D_{95}$ values obtained are shown in Table 6.

Alpha toxin activity did not diminish in either sample during the first 6 minutes of heating. Even after 18 minutes of heating, Sample A (Control) exhibited only a 29.6% loss of activity. However, Sample B upon heat treatment beyond 6 minutes became more susceptible to heat inactivation and proteose-peptone no longer afforded heat protection. The $D_{95}$ value decreased from 123.4 min. to 49.5 min. when Ellman's reagent was added.

Alpha toxin lost most of its activity after being heated at 95°C for 18 min. in the presence of NEM, whereas 43% of the activity remained when Ellman's reagent was present. This may be due to the reaction of NEM with other groups besides the -SH groups. Smyth et al. (1960)
Table 6. Effect of Ellman's reagent on heat inactivation of α-toxin in presence of proteose-peptone.

<table>
<thead>
<tr>
<th>Heating time (min)</th>
<th>% Residual Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample A (Control)</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>6</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>12</td>
<td>83.3 ± 1.5</td>
</tr>
<tr>
<td>18</td>
<td>71.4 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>$D_{95} = 123.4 \text{ min}$</td>
</tr>
</tbody>
</table>

(1) $D$ value was calculated from the 18 min. heating time.
(2) Average of two trials.
reported that NEM may react with imidazole and with the amino group of peptides. They suggested the need for caution in the use of NEM for the detection of sulfhydryl compounds in mixtures derived from biological sources or formed in enzymatic reactions.

**Effect of Calcium**

Proteose-peptone which is a pancreatic digest of casein, contains calcium. Calcium can form a bridge between negatively charged groups of proteins and cause the formation of a complex.

In order to investigate the possibility of calcium being involved in the complex formation, calcium was bound with the chelating agent, EDTA. To 5 ml of 2% proteose-peptone, 1 ml of 50 mM EDTA was added. The mixture was left to react at room temperature for 1 hour. It was then dialyzed for 48 hrs against distilled water to remove excess EDTA. Alpha toxin was then suspended in the EDTA treated proteose-peptone medium and heated at 95°C for 18 min. After this heat treatment, the percent residual activity of alpha toxin was 32.6%. When calcium chloride was added at 0.01 M concentration, the percent residual activity increased to 77% after heating at 95°C for 18 min. It is evident that the presence of calcium plays a role in the heat protection of alpha toxin. Whether or not a complex formation through calcium bridges was involved requires further study.

**Effect of Ionic Strength**

In order to ascertain the possible role of ionic strength on the heat inactivation of alpha toxin, the toxin was prepared in solutions containing different concentrations of NaCl i.e., 0.025, 0.05, 0.1, and 0.2 M. The toxin was diluted 1:64 v/v in the above salt solutions after
which 0.05 ml aliquots were heated for 0, 6, 12, and 18 min. at 95°C. Results obtained are shown in Table 7.

At high ionic strength, less alpha toxin was destroyed, probably due to "salting in" phenomenon which prevents gross aggregation through electrostatic repulsion of molecules having the same charge.

**Effect of Trypsin**

The effect of trypsin on the activity of alpha toxin and on the heat inactivation of alpha toxin was determined. This was done because Park (1976) found that a decline of alpha toxin activity occurred at the end of the exponential growth phase. This was thought to be due to degradation of the alpha toxin by proteolytic enzymes produced by the organisms. Shemanova et al. (1970) also demonstrated a decrease in phospholipase C activity in the culture supernatant which coincided with the maximum proteolytic activity.

A stock solution of trypsin (0.1%) in phosphate buffer, pH 7.6 was prepared. The activity of trypsin supplied by the manufacturer, was 11,000 BAEE units/mg powder.

First, the effect of trypsin on alpha toxin when suspended in proteose-peptone was determined. Alpha toxin was diluted in proteose-peptone (v/v). One-half milliliter of a proteose-peptone-alpha toxin solution was mixed with an equal volume of the trypsin solution. After mixing, 5 μl of the mixture was spotted on HI plates which were then incubated at 37°C for 24 hr. Residual activity of alpha toxin was 59.3% when compared to the control prepared without trypsin. An indication that trypsin influenced alpha toxin.
Table 7. Effect of ionic strength on heat inactivation of alpha toxin at 95°C for 18 min.

<table>
<thead>
<tr>
<th>Ionic Strength</th>
<th>0.2M</th>
<th>0.1M</th>
<th>0.05M</th>
<th>0.025M</th>
</tr>
</thead>
<tbody>
<tr>
<td>D_{95}</td>
<td>22.1 ± 2.5</td>
<td>12.3 ± 0.6</td>
<td>10.6 ± 0.4</td>
<td>15.2 ± 0.6</td>
</tr>
<tr>
<td>% Residual Activity</td>
<td>18.6 ± 2.8</td>
<td>3.4 ± 7</td>
<td>1.9 ± 0.3</td>
<td>0.87 ± 0.6</td>
</tr>
</tbody>
</table>

(1) Average of 4 trials with standard deviation.
(2) Standard curves are shown in Fig. 20-23, Appendix.
In order to determine whether the proteolytic action of trypsin on proteose-peptone also affected its heat protection of alpha toxin, the following experiment was performed: to 0.5 ml of a proteose-peptone solution, 0.5 ml of trypsin was added. Immediately after mixing, the mixture was heated at 75°C for 20 min. in order to inactivate the trypsin.

The temperature of trypsin inactivation was previously determined after heating trypsin at different temperatures and then assaying its proteolytic activity by the Azocoll method (Calbiochem, LaJolla, CA). Alpha toxin was then added to the trypsin treated proteose-peptone solution. This was followed by heat treatment at 75°C for 20 min.

The percent of residual activity compared to the control (alpha toxin in proteose-peptone, but not treated with trypsin) was found to be 30 (Table 8). Proteose-peptone, after being treated with trypsin, lost its protective effect to a great degree. This would be additional evidence that high molecular weight components are essential for the protective action of proteose-peptone against heat inactivation of alpha toxin.

When alpha toxin was treated with trypsin and then heated in phosphate buffer, pH 7.4, no residual activity was detected (Table 8). When the mixture of alpha toxin-proteose-peptone was treated with trypsin and then heated at 75°C for 20 min, the percent residual activity of alpha toxin compared to the control (alpha toxin in proteose-peptone heated at 75°C for 20 min, but not treated with trypsin) was a very low 0.4%. These data suggest than when alpha toxin is subjected to proteolysis it
Table 8. Effect of trypsin on heat inactivation of α-toxin in proteose-peptone (PP) and buffer systems.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Residual Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-toxin in PP + trypsin mix-spot immediately</td>
<td>58.3 ± 2.3</td>
</tr>
<tr>
<td>Proteose-peptone + trypsin mix-heat-add α-toxin-heat at 75°C for 20 min.</td>
<td>30 ± 1.5</td>
</tr>
<tr>
<td>α-toxin + PP + trypsin mix-heat at 75°C for 20 min.</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>α-toxin + phosphate buffer, pH 7.4 + trypsin mix-heat at 75°C for 20 min.</td>
<td>0</td>
</tr>
</tbody>
</table>

(1) Average of 4 trials with standard deviation.
becomes very susceptible to heat destruction and to a decrease in activity. The destruction is complete when alpha toxin is suspended in buffer rather than in proteose-peptone.

**Effect of Proteose-Peptone on Reactivation**

The effect of proteose-peptone on the reactivation of heat treated alpha toxin was studied next. This was done because the reactivation phenomenon by culture media has been reported for other unrelated toxins (Fung et al., 1973; Reichert and Fung, 1976).

Alpha toxin was prepared 1:16 v/v in saline. Aliquots (0.05 ml) of the solution were heated in thin wall glass tubes (10 x 75 mm) at 55, 75 and 95°C for 0, 6, 12, and 18 min. After heat treatment, samples were handled in 2 different manners as diagrammed below:

**Sample A**
- α-toxin in 0.85% NaCl (1:16)
- heat
- 0.85% NaCl added to a final dilution 1:64
- incubate for 24 hrs at 37°C
- Run HI Assay

**Sample B**
- α-toxin in 0.85% NaCl (1:16)
- heat
- add (1%) proteose-peptone to a final dilution 1:64
- Run HI Assay

After incubation of the HI plates, the diameter of the zones was measured and the toxin concentration in HU/ml was read from a standard curve. Results are shown in Table 9 and Fig. 13 and 14.
Table 9. Reactivation of alpha toxin in saline (Sample A) and in proteose-peptone (Sample B) following heat treatment of the alpha toxin at three different temperatures.

<table>
<thead>
<tr>
<th>Heating time and temperature</th>
<th>Control</th>
<th>Sample A&lt;sup&gt;(1)&lt;/sup&gt;</th>
<th>Sample B&lt;sup&gt;(2)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCl (HU/ml)</td>
<td>Reactivation %</td>
<td>Proteose-peptone (HU/ml)</td>
</tr>
<tr>
<td>55°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>0.36</td>
<td>0</td>
<td>0.36</td>
</tr>
<tr>
<td>6</td>
<td>0.36</td>
<td>0</td>
<td>0.36</td>
</tr>
<tr>
<td>12</td>
<td>0.29</td>
<td>0</td>
<td>0.36</td>
</tr>
<tr>
<td>18</td>
<td>0.26</td>
<td>3.84</td>
<td>0.36</td>
</tr>
<tr>
<td>75°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>0.36</td>
<td>0</td>
<td>0.36</td>
</tr>
<tr>
<td>6</td>
<td>0.13</td>
<td>7.69</td>
<td>0.17</td>
</tr>
<tr>
<td>12</td>
<td>0.07</td>
<td>1.35</td>
<td>0.15</td>
</tr>
<tr>
<td>18</td>
<td>0.04</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>95°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>0.35</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>6</td>
<td>0.15</td>
<td>0</td>
<td>0.19</td>
</tr>
<tr>
<td>12</td>
<td>0.09</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>18</td>
<td>0.05</td>
<td>0</td>
<td>0.09</td>
</tr>
</tbody>
</table>

<sup>(1)</sup>Physiological saline.

<sup>(2)</sup>Physiological saline with proteose-peptone added after heating.

<sup>(3)</sup>Average of 2 trials.
Figure 13. Reactivation of α-toxin by proteose-peptone. Solid lines, α-toxin heated in saline and then suspended in saline, and dotted lines, α-toxin heated in saline and then suspended in proteose-peptone.
Figure 14. Effect of proteose-peptone on reactivation (1-4) of alpha toxin upon heat treatment in physiological saline, (5-8). Heating time was: 0, 6, 12, and 18 min.
It is evident from the data that in the presence of proteose-peptone reactivation was after heating at 75°C. Reactivation at 95°C, although somewhat less than at 75°C, was still evident with 72% of the alpha toxin being reactivated after 18 min. heating. Some reactivation occurred in saline, but this was minor when compared to that observed with proteose-peptone.

No reactivation occurred in saline after heating at 95°C.

Surface Denaturation of Alpha Toxin

Macfarlane (1941) reported that alpha toxin lost its activity after agitation or exposure to the air in thin films indicating that alpha toxin is susceptible to surface denaturation. In this study, an attempt was made to ascertain the susceptibility to surface denaturation of alpha toxin suspended in physiological saline and in proteose-peptone.

Air was bubbled at a constant pressure (0.5 atm) into 5 ml of an alpha toxin solution. Activity by the HI method was determined after exposure intervals of 0, 5, 10 and 15 min. In order to make certain that the loss of activity was due to surface denaturation and not to oxidation, the experiment was repeated by bubbling nitrogen gas instead of air.

Surface denaturation was observed in all systems (Fig. 15). There was a difference, however, between the degree of surface denaturation of alpha toxin when suspended in physiological saline or proteose-peptone. Proteose-peptone protected alpha toxin against surface denaturation. This degree of protection was not as pronounced as was its heat protection property. Further, there was no difference between the effect of air bubbling or nitrogen bubbling on the percent residual activity of alpha toxin.
Figure 15. Surface denaturation of alpha toxin by air or nitrogen gas bubbling.
Residual Activity

- Air
- Nitrogen gas

0.85% NaCl
1% Proteose peptone

% Residual Activity vs. Time of bubbling min

Figure 15
Spectrophotometric Assay of Alpha toxin - Km Determination

The spectrophotometric assay of Kurioka and Matsuda (1976) was used to determine the Michaelis-Menten constant (Km) for the purified alpha toxin. This method utilizes p-nitrophenyl phosphorylcholine (NPPC) as a substrate and measures production of p-nitrophenol after hydrolysis by phospholipase C. The yellowish color formed can be followed spectrophotometrically at 440 nm. The extinction coefficient of p-nitrophenol is 15400 cm⁻¹M⁻¹.

The reaction mixture consisted of 2.3 ml of 0.25 M Tris-HCl buffer, pH 7.2, containing 60% sorbitol w/w and 0.2 ml of pure alpha toxin containing 0.04 mg of protein. The substrate concentrations were 5, 10, 20, 40, and 60 mmoles. The reaction was run for 90 min. at 35°C. The percent transmittance was read at 410 nm and converted to optical density from appropriate tables. The concentration of the product was calculated by the formula

\[ C = \frac{O.D.}{E} \]

where C = concentration, O.D. = optical density and E = extinction coefficient, e.g. O.D. = 0.051.

\[ C = \frac{0.051}{15,400} = 3.3 \times 10^{-6} \text{M} = 3.3 \mu \text{moles.} \]

The velocity was expressed as moles/min. of p-nitrophenol produced, e.g.

\[ \frac{3.3}{90} = 0.037 \text{ moles/min} = V_0. \]

Then \[ \frac{1}{S} \] and \[ \frac{1}{V_0} \] were calculated in order to prepare a Lineweaver-Burk plot. Results obtained are shown in Table 10 and Fig. 16.

The Km value was found to be 0.1 M (10⁻¹M) which is close to the 0.2 M reported by Kurioka and Matsuda (1976). This value is quite high and indicates low affinity of NPPC towards phospholipase C.
Table 10. Optical density, velocity and $1/v$ values obtained for purified alpha toxin by the spectrophotometric assay method.

<table>
<thead>
<tr>
<th>S (mmoles)</th>
<th>$\frac{1}{[S]}$</th>
<th>O.D.</th>
<th>p-Nitrophenol produced μ moles</th>
<th>$V_o$</th>
<th>$\frac{1}{\sqrt{V_o}}$</th>
<th>$K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.2</td>
<td>0.051</td>
<td>3.3</td>
<td>.04</td>
<td>26.5</td>
<td>0.1M</td>
</tr>
<tr>
<td>10</td>
<td>0.1</td>
<td>0.107</td>
<td>7.0</td>
<td>0.08</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.05</td>
<td>0.192</td>
<td>12.5</td>
<td>0.15</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.025</td>
<td>0.26</td>
<td>24.0</td>
<td>0.26</td>
<td>3.8</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.016</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 16. Lineweaver-Burk plot, based on data obtained by the spectrophotometric assay method.
DISCUSSION

Several of the different approaches utilized have failed to dissociate the hemolytic activity of the alpha toxin of \textit{C. perfringens} from its phospholipolytic activity. It must therefore be concluded that both of these activities reside with the same protein molecule.

There appear to be two differing views regarding the hemolytic and phospholipolytic activities of the alpha toxin of \textit{C. perfringens}. Bernheimer (1968) failed to separate the two activities by density gradient electrophoresis and isoelectric focusing. He concluded that both activities reside in the same molecule. Mitsui \textit{et al.} (1973) have also shown that both of these activities are associated with a single metabolic entity having a molecular weight of approximately 49,000. Our preparation of alpha toxin had a molecular weight of 45,000.

On the other hand, Sabban \textit{et al.} (1972) reported that \textit{C. perfringens} phospholipase C preparations can under certain conditions induce hemolysis of red blood cells without release of any organic phosphate from red blood cell membrane phospholipids. They observed that heating of the toxin in the presence of Ca$^{++}$ caused loss of the hemolytic activity, whereas hydrolytic activity was retained. These findings led them to conclude that hemolytic activity was distinct from hydrolytic activity. Whether or not these two activities were considered to be associated with two separate metabolites of \textit{C. perfringens} or with one protein molecule possessing multicatalytic sites was not reported.
For *Bacillus cereus*, there is convincing evidence that the hydrolytic and phospholipolytic activities reside in 2 different chemical entities. Attempts to separate these 2 activities from *B. cereus* preparations have been successful. Johnson (1967), using Sephadex gel filtration, demonstrated that hemolytic, phospholipolytic and lethal activities are catalyzed by separate proteins. Molnar (1962) reported the separation of *B. cereus* toxin into 2 components (Factors I and II) on a calcium phosphate gel column. The phospholipase activity, determined by the egg yolk reaction, was found in the fraction containing Factor I. Ivers and Potter (1977) have reported the hemolysin, phospholipase C, and lethal toxin of *B. cereus* are 3 different metabolites.

Before purification of the alpha toxin, the thermal inactivation time curve did not exhibit a definite negative slope throughout a heating range of 55-95°C when the toxin was suspended either in a proteose-peptone fraction or in saline. However, upon purification, the thermal inactivation time curve had a definite negative slope when the toxin was suspended in proteose-peptone. When the toxin was suspended in NaCl, the heat inactivation curve was broken above and below 65°C. A heat labile inhibitor may have been removed during purification which could have accounted for the differences observed in the thermal inactivation time curves. Manohar (1964) observed a similar phenomenon in heat studies of crude staphylococcal alpha toxin. He found that loss of alpha hemolytic activity of the toxin was more rapid at 60°C than at 100°C. This paradoxical behavior was postulated to be due to the presence of a thermolabile inhibitor in crude toxin.
The broken thermal inactivation time curve observed after heating purified alpha toxin in saline may be explained by the formation of low temperature aggregates which are disaggregated at higher temperatures. Jamlang et al. (1971) observed a similar phenomenon with staphylococcal enterotoxin B. Their explanation was that heat aggregation, which occurred at 70° and 80°C, was reversed at higher temperatures due to the low bond energy of the coagulation sites. The presence of proteose-peptone in our system might inhibit this aggregation.

Greenstein and Hoyer (1950) observed that deoxyribonucleic acid (DNA) prevents the heat coagulation of serum albumin. Nearly complete protection of albumin solutions against heat coagulation was afforded at ratios of approximately 100-200 mg of protein to 1 mg of DNA.

Studies on the protective effect of proteose-peptone against heat inactivation of alpha toxin indicated that this was due to a complex formed between the toxin and molecules of proteose-peptone. Both high and low molecular weight fractions were involved, the high molecular weight fractions being necessary for heat protection. The complex formation probably prevents the toxin molecule from further unfolding and thus inhibits the formation of gross aggregates.

Although only limited formation is available about the heat inactivation of the alpha toxin of C. perfringens and the mechanisms involved, research with toxins of other organisms, particularly those of S. aureus, would support our findings. We are aware of only one major study of the heat inactivation of C. perfringens alpha toxin. This is the study by Smith and Gardner (1950). They found that the rate of heat inactivation of alpha toxin was more rapid when the enzyme was
heated at 60-70°C than 80-90°C. Their explanation for this anomalous behavior was that the enzyme might be associating at the lower temperatures to form an inactive complex, probably through calcium and magnesium ions.

Work done by Saterlee and Kraft (1969), on the heat inactivation of staphylococcal enterotoxin B in a phosphate saline buffer, demonstrated that a crude enterotoxin B preparation was more heat stable than one which had been partially purified. This heat stability might be expected since the presence of other proteins, which are very abundant in the crude preparations, would give stability and protection to the enterotoxin B molecule.

Tatini (1976) studied the thermal stability of staphylococcal enterotoxins in food and found that enterotoxin A or B heated at 100°C for 24 min. in saline containing 2% gelatin and 0.3% proteose-peptone at pH 7.0 retained biological activity towards humans with a suggestion of increased biological activity after the heat treatment.

Lee et al. (1977) found that high D values were obtained when staphylococcal enterotoxin B was heated in beef broth and the protection afforded was in direct proportion to the protein concentration. In addition to the general protective effect of protein, there was a protective effect associated with a dialyzable fraction of beef broth protein. This led to the assumption that an interaction between a factor in the dialysate and staphylococcal enterotoxin B molecules played a major role in increasing the heat stability of staphylococcal enterotoxin B.

Donavan and Beardslee (1975) studied the heat stabilization produced by protein-protein association using a differential scanning colorimeter. They reported that when bovine α-trypsin complexed with soybean trypsin
inhibitor the denaturation temperature increased 19°C. However, the association of lysozyme with ovalbumin, which is weak and is charge-induced, had no effect on the heat stability of ovalbumin.

Donavan and Ross (1973) found that when biotin is bound to avidin, a temperature increase of 42°C is required to obtain a rate of denaturation equal to that found for avidin without biotin.

From the experiments with NEM and Ellman's reagent, it becomes apparent the disulfide interchange reaction is involved in the complex formation as shown in the following equation:

\[ \text{RSSR} + R'SH \overset{\text{RSSR} + RSH}{\rightleftharpoons} \]

The reaction is strongly catalyzed by thiols in neutral or alkaline solutions and is considered to be initiated by traces of mercaptide ions:

\[ \text{RSSR} + R'S \overset{\text{RSSR} + RSH}{\rightleftharpoons} \]
\[ \overset{\text{RSSR} + R'S}{\rightleftharpoons} \]

It is inhibited by SH reagents such as NEM, chloromercuribenzoate (CMB) and Ellman's reagent. The pH conditions of the proteose-peptone solution were ideal.

The disulfide interchange reaction has been reported as the reaction involved in the complex formed between β-lactoglobulin and casein upon heating. The formation of a complex between β-lactoglobulin and casein is prevented by SH blocking agents (Trautman and Swanson, 1959). Proteose-peptone is a pancreatic digest of casein. Thus, when the casein is degraded by proteolytic action, the SH groups are unmasked and become readily available for the disulfide interchange reaction.
Calcium was also found to be essential for the heat stabilization of alpha toxin by proteose-peptone. Calcium may contribute to a secondary complex formation between proteose-peptone and alpha toxin inhibiting gross aggregation of alpha toxin. Morr and Josephson (1969) reported that calcium plays a role in the interaction of casein and whey proteins through non-specific calcium linkages. This interaction may be more important than the thiol-disulfide type of interaction in stabilizing the whey protein components against gross heat aggregation.

Studies on the reactivation of alpha toxin by proteose-peptone have shown that proteose-peptone, when added to a heated mixture of alpha toxin-physiological saline, induced reactivation of alpha toxin.

During heat inactivation of alpha toxin, some molecules totally unfold, some partially unfold, and some molecules remain intact. Proteose-peptone possibly helps the partially unfolded molecules to reassociate and regain their activity. Reichert and Fung (1976) found that beef broth and Brain Heart Infusion provided noticeable recovery of staphylococcal enterotoxin B activity. In many cases, it has been demonstrated that denaturation is not an "all or nothing" phenomenon.

Findings on the effect of trypsin on the activity and on the heat inactivation of alpha toxin have indicated that trypsin causes some inactivation of alpha toxin increasing its susceptibility to heat inactivation. Inactivation of alpha toxin by trypsin, a proteolytic enzyme, may explain the rapid decline of alpha toxin activity in the culture medium after the organism has reached the stationary phase observed by Park (1976). Proteolytic enzymes are known to be produced by C. perfringens.
Experiments on the surface denaturation of alpha toxin have shown that alpha toxin is susceptible to surface denaturation. The degree of denaturation was higher when the toxin was suspended in physiological saline rather than in a 1% proteose-peptone solution. The term "surface denaturation" has been given to modifications undergone by protein molecules when they are located at gas-liquid, liquid-liquid, or solid-liquid interfaces. For some proteins, this denaturation is completely reversible, for others, it is irreversible (Joly, 1965).

Dietrich (1962) observed that lysozyme was protected against ultrasonic inactivation at least for a limited period of time by amino acids or peptides such as methionine and glutathione. The effect was due to stabilization of the molecular structure by protein binding of the added compound. The same situation may occur in the case of alpha toxin and proteose-peptone. However, it is more likely that protection of alpha toxin against surface denaturation by proteose-peptone involves competition for available surface at the gas-liquid interface.

Many of our findings have application to routine laboratory use of alpha toxin level as an index of C. perfringens population levels. Some of these factors are discussed below.

First, because the hemolytic and phospholipolytic activities of alpha toxin reside in the same protein, either the hemolytic agar plate test or the lecithovitellin test can be used as an assay procedure. Park and Mikolajcik (1977), however, have demonstrated conclusively that the HI plate test has greater sensitivity than the LV method.

Second, complex formation by means of S-S bonds or calcium bridges, apparently conveys a degree of heat resistance to the alpha toxin. Thus,
food systems which have received heat treatments not exceeding $95^\circ$C for 18 min. can be assayed for the presence of alpha toxin. The complex formation does require -S-S- -SH groups for heat protection. These are present in foods rich in proteins and peptides, foods which are involved in \textit{C. perfringens} food poisoning outbreaks.

Third, the sensitivity of the toxin to surface denaturation shows that caution must be used and especially during extraction procedures to avoid excess agitation.

Fourth, the sensitivity of the toxin to the proteolytic action indicates that data obtained when alpha toxin is used to detect previous growth of the organism must be interpreted with caution. A negative test for alpha toxin activity does not always mean the absence of the organism. The toxin activity could have been destroyed by proteolytic action of protease elaborated by organisms present in a food.

And, finally, if the alpha toxin procedure is to be applied routinely, the need now is to institute studies of the behavior of the \textit{C. perfringens} organism and the toxin in native food systems.
SUMMARY AND CONCLUSIONS

The overall objective of the research was to examine critically physical, chemical, and biochemical factors affecting the activity of *Clostridium perfringens* alpha toxin. An understanding of these factors is necessary if, as suggested, alpha toxin is to be used for estimation of the number of *C. perfringens* organisms present in a food system.

In line with the major objective, investigations were undertaken (a) to develop procedures for the preparation of highly purified alpha toxin; (b) to determine whether phospholipolytic and hemolytic activities of *C. perfringens* alpha toxin are associated with the same protein molecule; (c) to examine the mechanisms involved in heat protective properties for alpha toxin by proteose-peptone; (d) to determine the effect of surface denaturation on the activity of alpha toxin; (e) to explore the possible reactivation of alpha toxin in the presence of proteose-peptone; (f) to ascertain the influence of proteolytic enzymes on the activity and heat inactivation of alpha toxin; (g) to determine the effect of ionic strength on heat inactivation of alpha toxin; and (h) to establish the Michaelis-Menten constant, $K_m$, for highly purified alpha toxin.

For purification and isolation of alpha toxin, gel filtration on Sephadex G-100 and polyacrylamide disc gel electrophoresis were used. The purity of the preparations was determined by immunodiffusion,
immunoelectrophoresis, and disc gel electrophoresis with sodium dodecyl sulfate. Complex formation between alpha toxin and proteose-peptone and its role in heat protection were examined by means of gel filtration on Sephadex G-75 column. The type of reaction involved in the complex formation was studied by use of Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)], NEM (n-ethylmaleimide), and Ca^{++} ions. The influence of proteolytic enzymes on alpha toxin was investigated by the use of trypsin. For the determination of surface denaturation, air or nitrogen gas was bubbled in preparations of alpha toxin suspended in NaCl or proteose-peptone. The effect of ionic strength on the heat inactivation of alpha toxin was studied using suspensions of alpha toxin in solutions containing different salt solutions. The Km was determined using a spectrophotometric assay method introduced by Matsuto and Kurioka (1976).

The results of the study would support the following conclusions:

1. Both phospholipolytic and hemolytic activities of C. perfringens alpha toxin were shown to reside with the same protein molecule.

2. High and low molecular weight components of proteose-peptone protected alpha toxin against heat inactivation by means of complex formation.

3. The -S-S -SH disulfide interchange reaction was involved in complex formation.

4. Calcium also afforded heat protection.

5. Proteose-peptone reactivated heat treated alpha toxin upon incubation at 37°C for 24 hrs.
6. Alpha toxin was susceptible to surface denaturation.

7. Proteose-peptone protected alpha toxin to a limited extent against surface denaturation.

8. There was no discernible difference between surface denaturation produced by air or nitrogen gas.

9. Alpha toxin activity was affected by the proteolytic enzyme, trypsin.

10. Trypsin increased alpha toxin susceptibility to heat inactivation.

11. Trypsin treatment of proteose-peptone per se reduced its heat protective properties for alpha toxin.

12. Alpha toxin was more susceptible to heat inactivation at low ionic (0.05 M) than high ionic (0.2 M) strength salt solutions.

13. The Km value for purified alpha toxin, as determined by the spectrophotometric procedure, was 0.1 M.
Figure 17. Standard curve for LV test run in buffer, based upon 7 trials. Confidence limits are shown on curve (p = 0.05).
Figure 17

Log LV units/ml vs Zone diameter mm

$r = 0.960$

±0.396

±0.259

±0.396

Zone diameter mm
Figure 18. Standard curve for HI test run on alpha toxin suspended in proteose-peptone. Data are for 6 trials with confidence limits shown (p = 0.05).
Figure 18

Log HU/ml vs. Zone diameter mm

$R = 0.987$
Figure 19. Standard curve for HI test run on alpha toxin suspended in physiological saline. Data are for 10 trials, with confidence limits shown (p = 0.05).
Figure 19

Log HU/ml vs Zone diameter mm

$r = 0.982$

$\pm 0.161$

$\pm 0.279$

$\pm 0.278$
Figure 20. Standard curve for HI test run on alpha toxin suspended in 0.2 M NaCl. Data are for 3 trials with confidence limits shown (p = 0.05).
Figure 20

Log HU/ml vs. Zone diameter mm

\[ r = 0.972 \]

Figures: +0.435, +0.252, +0.436
Figure 21. Standard curve for HI test run on alpha toxin suspended in 0.1 M NaCl. Data are for 3 trials with confidence limits shown ($p = 0.05$).
Figure 21

Log HU/ml vs Zone diameter mm

$R = 0.986$

$\pm 0.340$

$\pm 0.197$
Figure 22. Standard curve for HI test run on alpha toxin suspended in 0.05 M NaCl. Data are for 3 trials with confidence limits shown (p = 0.05).
Figure 22

Log HU/mm vs Zone diameter mm

\[ r = 0.989 \]
Figure 23. Standard curve for HI test run on alpha toxin suspended in 0.025 M NaCl. Data are for 3 trials with confidence limits shown (p = 0.05).
Figure 23
REFERENCES


