ABSTRACT

STUDIES ON CLOSTRIDIUM PERFRINGENS
TYPE C TOXIN INJECTED INTRAVENOUSLY
IN DAIRY CALVES

by John R. Welser

Clostridium perfringens Type C (beta) toxin was obtained from the research division of a veterinary pharmaceutical company* for experimental work on the bovine species.

Intravenous injection into white mice (17-20 Gm.) was used to check the type and minimal lethal dose of the toxin. White mice were also used to determine the antitoxin level in calves' sera, to determine how long the toxin remained in the blood stream of the calves, and to determine if the urine voided from the calves following toxin administration contained a lethal factor.

In preliminary work, it was found that the toxin would not kill calves, but would kill goats and mice. The routes of administration tried in calves were intravenous, oral, and direct injection into the intestinal tract. However, if the toxin was combined with trypsin, death could be produced.

Six calves, whose sera was negative for antitoxin, were injected intravenously at the rate of 15,000 mouse MLD/100 pounds body weight. The symptoms shown following

administration of the toxin were: coughing, lacrimation, colic, urination, slight respiratory distress, temperature increase of one degree, doubled heart and respiratory rate, decrease in packed cell volume, increase in the total leukocyte count, and an inverse relationship between the numbers of neutrophils and lymphocytes.

Sera samples taken from the jugular vein of six calves after intravenous injection of the toxin and injected into mice showed that the toxin was removed from the calves' blood stream within twenty-five minutes.

A factor lethal to mice appearing in the calves' urine collected two to three hours after toxin injection suggests that the toxin may be excreted in a relatively unchanged form.

---

*Haver-Lockhart Laboratories, Kansas City, Missouri: Dr. F. W. Binkley, research clinician. Toxin was originally sent to Dr. G. R. Moore, Director of large animal clinics, Michigan State University.

STUDIES ON *CLOSTRIDIUM PERFRINGENS* TYPE C TOXIN INJECTED INTRAVENOUSLY IN DAIRY CALVES

By

John R. Welser

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Surgery and Medicine

1962

ACKNOWLEDGMENTS

My sincere appreciation is extended to all those who so willingly contributed their time and interest in making this study possible.

Special notes of thanks are offered to the following people: Dr. G. H. Conner, Professor, Department of Surgery and Medicine, for his physical help, guidance, counseling and especially, his encouragement; Dr. M. P. Rines, Assistant Professor, Department of Surgery and Medicine, for his constructive criticism, disciplined thinking, and advice; Dr. G. R. Moore, Professor and Director of Large Animal Clinics, Department of Surgery and Medicine, for providing the facilities, materials, ideas, and for his cooperation; Dr. D. J. Ellis, Assistant Professor and Director of Farm Services, Department of Surgery and Medicine, who was a source of inspiration and evaluation; and Dr. C. C. Beck, Assistant Professor, Department of Surgery and Medicine, for his stimulating ideas and reference material.

The author is also deeply indebted to Dr. W. O. Brinker, Professor and Head, Department of Surgery and Medicine, for his helpful interest and guidance. Recognition is due to Dr. Virginia Mallman and Mrs. Marian Bennett

for their knowledge and advice concerning the bacteriological aspects of this study.

Haver-Lockhart Laboratories (Kansas City, Missouri) and Dr. F. W. Binkley, Director of Research, provided the toxin and suggestions which were necessary to initiate my research. To them and to all others who contributed in any manner, my sincere thanks.

TABLE OF CONTENTS

LIST OF TABLES

TABLE Page

LIST OF CHARTS

PART I

INTRODUCTION

The clostridial group of organisms are responsible for many of the more serious disease maladies of domestic animals. Included in a list of the common diseases caused by them would be blackleg, tetanus, malignant edema, enterotoxemia, botulism, red water, and infectious necrotic hepatitis. In foreign countries, people refer to black disease, struck, braxy, lamb dysentery and pulpy kidney, each caused by a species of the clostridia (22). This study deals with just one of the clostridia--Clostridium perfringens, type C and its toxin.

According to the literature, the toxin produced by Clostridium perfringens, type C, is responsible for enterotoxemia in cattle, a disease characterized by sudden death. The amount of toxin necessary for death is not known, nor are the symptoms in cases where cattle are subjected to sub-lethal doses of toxin.

This project was originally designed to determine the minimal lethal dose and symptoms observed of type C toxin in calves. Later the design was modified to include the toxin's effect in blood and urine as well as the length

of time necessary for the toxin to disappear from the blood of calves following its intravenous administration.

PART II

REVIEW OF LITERATURE

History:

In veterinary medical literature, the term enterotoxemia was apparently first used by Bennetts in 1932 to describe an acute disease of sheep. Later acute hemorrhagic enteritis was observed in young, suckling range calves for a number of years in Colorado. After several erroneous diagnoses were made, the disease was specifically identified and the term enterotoxemia applied to it (12).

Various other names have been given to this condition in calves, most of them based on the gross pathology. Among the more common are acute hemorrhagic enteritis, bovine hemorrhagic enterotoxemia, and hemorrhagic enterotoxemia (12, 26). Merchant defines the condition as follows: "Bovine hemorrhagic enterotoxemia, an acute disease of young calves, lambs, and sheep, caused by Clostridium perfringens Type C (beta toxin), characterized by sudden onset, hemorrhagic enteritis and early death (26)." Griner, who has done considerable work in this field, describes it as follows: "Acute hemorrhagic enteritis, a newly recognized infectious disease resembling enterotoxemia of lambs, seen

in young suckling range calves in Colorado, characterized by acute, sudden onset, hemorrhagic enteritis, severe hemorrhage into the lumen of the small intestine and early death (17)."

Clostridium perfringens has previously been designated as Clostridium welchii, Clostridium ovitoxicus, Bacillus phlegmones-emphysematosae, Welch bacillus, and gas bacillus. It was originally isolated in 1892 from the foamy organs of a cadaver by Welch and Nuttall, and called Bacillus aerogenes capsulatus (21, 37). In 1898, Veillon and Zuber called it Bacillus perfringens; it was then referred to as Clostridium welchii in English speaking countries, and as Clostridium perfringens in France. In 1930, it was designated Clostridium perfringens by Bergey (21, 7).

Morphology:

Clostridium perfringens is a large, thick, straight-sided, encapsulated, non motile, gram-positive rod occurring either singly or in pairs, but seldom in chains. Individual cells are about 1 micron wide and from 4-8 microns long. The spores are oval, small, and cause little swelling of the rods. The rods vary in their ability to sporulate, and spores will not form in a highly acid medium. Neither are they apt to be found in media that contain fermentable carbohydrates (7, 21, 37). The formed spores are not highly resistant, being destroyed at 100 C. in less

than 5 minutes (21).

The bacillus is a capsulated, strict anaerobe that grows readily in deep brain meat infusion broth, agar and gelatin media. On agar plates, round, entire, slightly raised, opaque center colonies are formed, varying from smooth convex discs with an unbroken edge to an umbonate rough colony with a crenated edge. The colonies are surrounded by a zone of hemolysis on blood agar plates. Clostridium perfringens will form small, biconvex colonies in deep agar, and if fermentable sugar is present, the media will fragment due to gas formation (7, 21, 37).

Clostridium perfringens will produce acid and gas in glucose, maltose, lactose, levulose, galactose, mannose, and sucrose broth. Neither mannitol nor salicin is fermented and the fermentation of inulin and glycerol is variable. Gelatin is rapidly liquefied, but coagulated egg medium and Leoffler's blood serum are not liquefied by the bacillus. In broth cultures, excellent growth takes place as the media become cloudy. Good growth with gas formation is observed in cooked meat medium; however, the meat fragments are not digested. Litmus milk shows stormy fermentation, coagulation, and acid with gas formation. Hydrogen sulfide is produced by Clostridium perfringens while indole is not (7, 21, 37).

Stormy fermentation of milk, non-motility, and wide distribution in feces, sewage and soil are considered by

Bergey's Manual to be the distinguishing characteristics of Clostridium perfringens (7).

Classification and Conditions Caused:

Clostridium perfringens, the most prolific toxin producer of the clostridia, is divided into 6 types, A through F. From these 6 types, 15 soluble antigenic toxic fractions have been identified and designated by letters of the Greek alphabet (17,38). Oakley and Warrack indicate that each of the 6 types produce one or more antigenic fractions. Generally, the one in greatest quantity is termed major and the others are termed minor (17, 42). Wilsdon (1931) first established the immunological relationship of the four main strains by comparing the antigenic properties of culture filtrates based on the presence of three lethal factors, which he called W, X, Z (17, 37, 42). In 1933, elaborating on Wilsdon's work, Clenny, Barr, Llewellyn, Jones, Dalling and Ross showed that Wilsdon's Z fraction was composed of at least 3 toxin components, and they proposed a Greek alphabet terminology (37).

Today the system is still termed the Wilsdon's classification and the soluble antigenic toxic fractions are classified by their activity on lecithin, hyaluronidase, desoxyribonucleic acids, ability to cause lysis of erythrocytes, necrosis following intracutaneous injection and death following intravenous inoculation (21). Identification is

based on toxin neutralization and determination of enzymatic activity (21). See C. L. Oakley and G. L. Warrack (30) for the methods used.

The following table gives the tabulation of the properties and distribution among the types of the known toxin soluble antigen of Clostridia perfringens (17, 30).

Tabulation of the Toxin Antigen of Clostridium perfringens Filtrates

Names	Activity Biological	Biochemical	Occurrence in filtrates of Clostridium perfringens types A	B	C	D	E	F
Alpha	Lethal, necrotic Hemolytic	Lecithinase	+++	+	+	+	+	+
Beta	Lethal necrotic	?		+++	+++			
Gamma	Lethal	?		+	+			+
Delta	Lethal hemolytic	?		+	++			
Epsilon	Lethal necrotic	?		++		+++		
Eta	Lethal	?	(+?)					
Theta	Lethal hemolytic	?	++	+	+	+	+	
Iota	Lethal necrotic	?					++	
Kappa	Lethal necrotic	Collagenase	++		+	±	+	
Lambda	?	Proteolytic enzyme		+		+	+	
Mu	Spreading factor affects capacity	Hyaluronidase		++	+			
Nu	of leukocytes to stain	Deoxyribonuclease	+	+	+	+	+	+

± = produced by some strains

+ & ++ = minor toxin

+++ = major toxin

Some strains of Clostridium perfringens may lose their capacity to produce particular antigens and are called degraded strains (30). Dalling and Ross, 1938, stressed that there are optimal cultural conditions for the production of each toxin, and unless these conditions are met, a parent strain may fail to yield a measurable quantity of one or the other toxin types (6). Likewise, laboratory toxicogenic types can be varied from poor to good by varying the media (1). Many times, this accounts for incorrect typing.

Toxins are believed to be products of bacterial metabolism. They consist of separate fractions having dual or separate specific action on certain cells or tissues (15). Oakley defines a toxin as a substance of high molecular weight, generally a protein, capable of damaging animal cells and possessing antigenicity which is capable, upon injection into a living animal, of exciting the production of substances called antitoxins; these are able to neutralize its toxic properties (28).

The ability of the bacillus to produce hyaluronidase, a spreading factor, may be linked with pathogenicity and rapid spreading of the infection. Collagenase, which is present in culture filtrates of Clostridium perfringens, is believed to be partly responsible for the solvent action on supporting connective tissue as well as the disintegration of muscle (15, 2).

According to Griner, localized necrotizing action of tissues is a feature of the toxin of Clostridium perfringens; gas production and odor which rise from the hydrolytic oxidative and deaminative action on affected tissues also result. The lethal effect is due to the combined effect of toxin components on vital tissues and cells of the host (15, 2).

Clostridium perfringens may be found either alone or mixed with other bacteria in diseases of animals. The different strains vary greatly in pathogenicity, but most will kill or produce disease in man and the domestic animals. As for the laboratory animals, most of the strains of Clostridium perfringens will kill mice, guinea pigs, pigeons and rabbits (21, 26). Since Clostridium perfringens is widespread in the soil and found in the alimentary tract of nearly all species of warm blooded animals, it is frequently found as a post mortem invader from the alimentary tract into the tissues of bloating cadavers of man and animals. For this reason, caution must be taken in drawing conclusions based on the presence of the organism in the tissues collected after death. It is found most often in the so-called gas gangrene infection of man (21).

The following is a list of the 6 toxigenic groups A through F of Clostridium perfringens, the conditions reported, history, and the species in which they occur:

Type A: 1936--Rose and Graham reported enterotoxemia jaundice of sheep and calves in Australia, resembling acute leptospirosis due to the hemolytic activity of A toxin (3, 12).

--gas gangrene in man and animals (17).

1943--enterotoxemia in young calves by Macrae, Murray and Grant in England (17).

--traumatic wound infection of animals (21).

1958--acute enterotoxemia in a 6-month old feeder steer (3).

--saprophytic-intestinal tract of all species (1).

Type B: 1923--Gaiger and Dalling recorded the isolation of lamb dysentery in England (37).

1925--Dalling, Allen, Mason showed the lamb dysentery bacillus to produce a lethal toxin (37).

1932--Gill reported pulpy kidney disease of lambs in New Zealand (37).

1937--Dysentery reported in foals by Montgomerie and Rowlands (23).

1938--Enteritis in foals by Mason and Robinson (23).

1952--Hepple reported necrotic enterotoxemia in calves (23).

1956--Frank of Wyoming reported enteritis in lambs and calves (9).

Type B produces 2 major toxin components, beta and epsilon (42). It should be noted that by suitable cultural methods, type B organisms could be made to produce almost pure beta or epsilon toxin (42). The capacity to produce toxin is readily lost by many strains of type B (9). Some believe it has not been definitely established as a disease

entity in the United States (9, 12).

Type C: 1930--Isolated by McEwen and Roberts as the cause of "struck" in adult sheep in England, largely restricted to the Romney Marsh (37).

1933--Heller gave an account of acute hemorrhagic enteritis in young shed born lambs in California (17).

1951--Study started at Colorado A and M (17) was observed and confirmed in numerous areas in Colorado after being found in 6 calves at autopsy (18).

1952--Acute hemorrhagic enteritis described by Griner and Bracken in 5 calves at autopsy (18).

1953--Griner and Johnson reported similar hemorrhagic enteritis of new born lambs (17).

1953--Excessive mortality of newborn lambs 12-72 hours after birth (20).

1953--Griner and Bracken reported hemorrhagic enteritis and reproduction of the disease by feeding a whole broth culture of Clostridium perfringens type C combined with cornmeal and milk (15).

1955--Field and Gibson reported enteritis in piglets in England (12).

1956--Barrons observed one case of enteritis in goats (19).

Type C produces a lethal necrotizing toxin isolated first by McEwen. A member of the Welch group, it was first designated Bacillus paludis, and later became type C in the Wilsdon classification, 1931 (17). It most commonly affects adult sheep, according to Griner (20).

Type D: 1931--Schofield reported enterotoxemia in cattle (24).

1932--In western Australia, Clostridium perfringens type D isolated from the small intestine of sheep as the cause of enterotoxemia (1).

1939--Gordon found type D toxin in Scotland in horses with grass disease (21).

1954--Keast and McBarron reported enterotoxemia in cattle in Australia (12, 16).

--enterotoxemia in sheep and goats throughout the world (12, 17).

Epsilon toxin is excreted as a protoxin. Upon standing, the toxin is converted to an active, highly lethal necrotizing toxin by proteolytic enzymes (38). This also can be accomplished by incubating the toxin at 37° C. with trypsin for one hour (22). This process increases the lethal quality of the toxin by approximately 300 times (22).

Type E: 1921--Gaiger and Dalling reported bacillary dysentery in lambs and reproduction by feeding the intestinal contents of a natural infected lamb (27).

1943--Bosworth observed hemorrhagic enteritis in calves 1-4 weeks old (12).

1954--Griner reported hemorrhagic enteritis in calves due to Type E.

Type F: --causes enterotoxemia of man called enteritis necroticans.

--characterized by the ability of spores to withstand boiling for 4 hours (3, 12, 17, 21).

In a review of the literature, it is noted that all of the types of Clostridium perfringens A through F have been reported as causing a condition in calves (3, 12). It also may produce a fulminating mastitis, but no attempt

at typing has been made (15).

From the above reports, it becomes obvious that little evidence of a species specificity for any of the six types can be found, and a clear cut classification is impossible (3, 12).

Type C:

Epizootiology:

Griner and Bracken reported that hemorrhagic enteritis occurred most often in vigorous thrifty young animals (12). This is confirmed by stockmen who rarely report losing a small weak calf. The animal lost is generally large, well formed and apparently vigorous (39). It occurs in calves 2-10 days of age having high producing cows as their dams (3, 12). Most of the other reports on the condition agree that it occurs in calves under 3 weeks of age (1, 3, 26, 40). However, Barner (2), along with Stableforth and Galloway (27) limit the disease to 3-5 days of age. Accordingly, it is generally agreed to occur most commonly in the beef breeds with the highest incidence being in the Hereford breed (15, 17, 26). Griner further states that few instances of the condition have occurred in the dairy breeds (17). However, Beck and Ellis (3) state that it does occur in dairy cattle with the highest incidence being the Holstein and Guernsey breeds.

Cattlemen agree that the most common predisposing factors listed include overeating and dams producing an abundance of milk (1, 3, 12, 15, 26). According to Griner, unfavorable cold, wet and windy weather at calving increases the incidence (17, 15). Baldwin reports an increase in incidence in the west when an increase in the number of spring storms is noted (1). However, Merchant reports there is no relationship to season, climate or weather (26).

Griner reports mortality in herds is 1-10% with unconfirmed reports up to 30%. Most stockmen are of the opinion that morbidity of the disease is greater than mortality. They believe that animals develop subacute infections from which they frequently recover but remain unthrifty (15). This is somewhat supported by Griner and Baldwin along with serological studies, which showed that 14% of the cows tested had normal antitoxin, and 24% of the calves had antitoxin (17). Baldwin reports that western cattlemen who have vaccination programs observed a drop in the incidence of debilitating diarrhea in calves (1).

Case History:

Due to the sudden onset and short course of enterotoxemia, the history is usually the finding of calves prostrate or dead (12). Beck and Ellis report the common history to be an animal seen normal one night or morning and upon the next observation by the owner, to be found dead (3).

Baldwin, however, reports a period of listlessness and inappetence 12-24 hours prior to onset (1).

There is usually a history of an excellent diet or a good milking dam (39). However, there is no agreement on the theory of an increase in incidence in calves from mature cows versus calves from heifers (2, 39). Reports appear in the literature of individual cows that have lost their calves for 2-3 consecutive years (12, 39).

Pathogenesis:

Enterotoxemia as a term was first used by Bennetts in 1932 to describe an acute disease of sheep caused by epsilon toxin of Clostridium perfringens type D. Since then, it has been applied to toxemia occurring in other species caused by Clostridium perfringens (12).

The pathogenesis of this disease is unknown (15). In the laboratory, anaerobic conditions, 37 C. temperature, and a media rich in protein and carbohydrate, gave maximum growth in four hours (1).

Clostridium perfringens is a saprophyte, normal in the lower intestine of all domestic animals. The fact that it is a spore former coupled with its saprophytic existence, gives a large population wherever livestock exist, thus making enterotoxemia an ever present possibility (1).

We know it is a pathogen, according to Beck and Ellis (3), from post mortem findings and animal tests with

toxin produced from cultures obtained from the small intestine of actual cases. Support is given to this by other examples of organisms that are residents under normal conditions and become pathogens; examples are streptococci, coliforms, and staphylococci (31).

Griner states that enterotoxemia is related to primary indigestion in an animal on a high carbohydrate ration or an increase in feed, or a sudden change in type of feed, which creates a favorable intestinal medium for the production of large quantities of toxin (12, 15). This is supported by Merchant who states: "Spores of the organisms are ingested from the environment; inflammation and engorgement of the bowel causes anaerobic conditions for the growth of the organism and the development of toxin. The intestinal stasis is conducive to absorption of toxin and its distribution by the blood stream. The toxin is produced in the intestine due to enteritis and probably overfeeding which causes the stasis and resulting absorption of toxin (26)."

Baldwin (1), Burns Co. Symposium (21), Bullen, and Batty (8), and Smith and Jones (35) all subscribe to the idea of overeating as the trigger mechanism. Baldwin claims, however, that the static condition of the bowel prevents flushing, and the rich feed provides the media (1). On the other hand, Burns Co. Symposium (21) along with Bullen and Batty, maintain it is due to an overflow of unfermented starch from the rumen into the small intestine. Roberts

claims the organism is markedly susceptible to acidity, and that overloading the rumen with high protein feed swamps the stomach acid allowing the organism to grow rapidly (37).

Beck and Ellis proposed that the trigger mechanism in the older cattle is stress as related to liver function (3). Their proposal is based on liver function and the role of bile in controlling Clostridium perfringens. Since bile is the alkaline reserve for proper pH in the body, and under conditions of stress the gall bladder does not empty properly, a more acid medium is created. The lack of bile gives decreased fat absorption; hence, food material in the gut becomes coated with fat, which results in protein putrifaction. Further support is gained from the fact that bile is used in bacteriology to inhibit gram positive organisms and a lack of it would tend to let them grow more profusely (3).

Bullen and Batty (8), in experimental work, found that when concentrated diphtheria antitoxin was dripped into the duodenum of normal sheep, small but constant amounts are absorbed into the blood, showing that the intestine is very slightly permeable to this protein. The rate of absorption of the antitoxin is not significantly affected by the sheep's overeating or by acid conditions in the rumen. However, in experimental enteritis, the rate of absorption is greater than normal, which shows that the permeability of the intestine is increased (8).

Likewise, massive quantities of the toxins of any of the enterotoxemia organisms can be fed to susceptible animals without evoking any signs of ill health. Therefore, it must be concluded that some unknown mechanism initiates the state of permeability, or more specifically, necrosis of the mucosa (37).

Toxin found in the peritoneal fluid but absent in the thoracic fluid, suggests a direct diffusion, but the missing link is an explanation of the permeability of the intestinal wall to the toxin (37).

Enterotoxemia has been reproduced in experimental sheep by the following ways: (1) previously injuring the lambs' alimentary tract; (2) functionally impairing it with opium and belladonna; (3) distending it with excessive amounts of milk and irritating it with a heavy feeding of cornmeal; (4) ligation of the jejunum and feeding culture (35). It has also been reproduced in sheep by feeding them whole broth cultures as well as intestinal contents of sheep that have died from the condition (27).

Not much success has been reported in reproducing enterotoxemia in calves. Griner reports one success out of four tries using whole broth culture of Clostridium perfringens (18). One other report in the literature of reproducing the condition, included the feeding of cornmeal or some other irritant food to the calves prior to feeding the culture of Clostridium (35).

Symptoms:

There is general agreement that the symptoms for Clostridium perfringens type C enterotoxemia vary depending on the severity of intoxication (1, 15, 39, 40) and range from a subacute form as recognized by Griner (12) to sudden death.

The acute form is often preceded by a period of 12-24 hours of listlessness, weakness and inappetance (1, 39, 26). Following this, acute colicy pains set in along with uneasiness, straining to defecate and kicking at the abdomen (1, 3, 15, 25, 39, 40). Hemorrhagic diarrhea may or may not occur depending upon the duration of the condition (1, 3, 26, 39, 40). Baldwin states: "If the animal lives over 6 hours, a hemorrhagic diarrhea is observed with the feces containing large quantities of fresh undigested blood (1)." When and if the bloody scours do appear, it generally indicates that the disease is in an advanced stage (15).

Just prior to death, the animal becomes prostrate, develops opisthotonus, tetanic spasms and toxic symptoms. The entire course of enterotoxemia is usually from 2-24 hours (1, 25, 26, 39, 40). The temperature remains normal throughout the course of the condition, becoming subnormal as the animal approaches death (15, 39).

The subacute enterotoxemia, as recognized by Griner, is characterized by diarrhea, listlessness and anorexia,

followed by acute colic, straining, and kicking at the abdomen (40). It somewhat resembles calf scours in that most animals recover but remain unthrifty (39). This opinion is supported by serological studies where the dams showed no antitoxin titer, although their calves had titers up to 16 units (12, 39). The two field case reports in the literature, one by Beck and Ellis (3) and the other by Gregory (10), support the above symptoms.

Necropsy:

In enterotoxemia, caused by type C of Clostridium perfringens, one can always observe an acute hemorrhagic enteritis of the jejunum and ileum, involving as much as from 20 consecutive feet to the entire small intestine (3, 15, 18, 19, 26, 39). Griner reports that in some cases, the enteritis is necrotic with desquamation of the mucosa (18). The lumen of the intestine has much free blood and necrotic tissue debris in it (3, 15, 39). Merchant states that a light fibrinous exudate covers the serosa of the intestine, but Griner describes it as a mild fibrinous peritonitis covering inflamed intestines (15). Extensive hemorrhage into the lumen and wall of the intestines was observed by Griner (15), and in another case, he reported subserosal hemorrhage along the entire digestive tract (18). This is confirmed by all other authors who observed petechial or ecchymotic hemorrhages throughout the remainder of the

digestive tract (3, 26, 39). Stableforth and Galloway (37) state that hemorrhages occur in the mesentery also.

Petechial or ecchymotic hemorrhages occur on the epicardium, thymus, diaphragm, abomassum and inconsistently on the parietal pleura (3, 12, 17, 26, 39). In addition, Griner reports subepicardial and endocardial hemorrhages (12). The mesenteric lymph nodes show serohemorrhagic lymphadinitis (3, 15, 26). Beck and Ellis (3) report that nearly all lymph nodes are swollen and hemorrhagic.

The peritoneal cavity contains a small quantity of serosanguinous fluid and an excess of fluid with some clotting is noted in the pericardial sac (15). Moderate pulmonary congestion was noted by Merchant (26). The abomassum is frequently distended with milk. Its mucous membrane is hyperemic and covered with a thick mucus (12, 15).

In the field cases, Beck and Ellis (3) observed a severe toxemia. A central nervous system involvement was substantiated by finding petechial hemorrhages on the brain and cord. The kidneys showed hemorrhages and the large intestine was distended with gas (3). Gregory (10) observed the following post mortem lesions: cecum distended with gas, the small intestines empty with a mucoid cast, and the walls of both the large and small intestines showing severe edema. The kidneys were highly congested with a soft friable parenchyma (10).

Histopathology:

Very little work has been done on the histopathology of type C enterotoxemia. Griner reports that most of the changes occur in the small intestine (15). Extensive necrosis and hemorrhage are the principal lesions occurring in the mucosa and submucosa of the small intestine with the necrotic process extending to the muscularis mucosae. Varying degrees of hemorrhage, vascular congestion, edema and distention of the lymphatics in the muscularis and subserosal occurs (17). Frequently, the villi are completely destroyed, presumably by the necrotic action of the toxin (17). Toxin degeneration of the liver and kidney parenchyma is consistently observed (15). The kidneys, myocardium, thymus and mesenteric lymph nodes show areas of hyperemia, hemorrhage and toxic degeneration (18). Hemorrhages in the perivascular spaces of the brain stem, as well as subepicardial and focal myocardial hemorrhages, may be observed (15, 18).

A smear of the lumen contents of the small intestine reveals large quantities of intact and hemolytic erythrocytes, fragments of necrosed villi, polymorphonuclear leukocytes and many gram positive rod shaped bacilli occurring singly or in short chains (15, 17, 18).

Comparisons: other species

A review of the literature concerning the symptoms,

pathology and histopathology of type C enterotoxemia in other species reveals a marked similarity to the condition occurring in calves. In sheep, the condition is characterized by sudden onset, early death, and post mortem lesions of a severe hemorrhagic enteritis (1, 17, 20). Shivering, bleating and other signs of chilling are shown as initial symptoms (17, 20). The rest of the symptoms and pathology are identical, except that there is general agreement that the condition is less severe in lambs (1, 20, 17).

More work has been done on the histopathology in lambs, and the lesions shown in liver and kidney sections are as follows:

A. Liver--central congestion and cloudy swelling of hepatic cords, swollen parenchymal tissue and pigmentation of cytoplasm.

B. Kidneys--hyperemia and hemorrhage in the parenchymal and stromal tissues, swollen epithelium of the convoluted tubules and granulated cytoplasm (17).

According to Stableforth and Galloway (37), the post mortem findings and pathologic changes seen in C enterotoxemia occurring in the English Romney Marsh area, differs from that described by Griner and Johnson in the United States (20).

Piglets showing hemorrhagic enteritis similar to calves have been observed in England and the United States.

The autopsy reports in both cases showed an acute necrotic hemorrhagic enteritis of the ileum and jejunum (1, 12).

Comparisons: other types

In the case of other types of Clostridium perfringens, A, B, D, E, causing enterotoxemia in calves, a striking similarity is noted. A short review of the lesions observed and the main difference is presented.

Type A. The symptoms and pathology observed in type A enterotoxemia are very similar to type C. Differences include: the presence of diarrhea, a temperature increase (103-106), more gas formation causing more gas to be found in the digestive tract, and a closer resemblance to acute leptospirosis due to the hemolytic activity of type A toxin (12, 25, 32, 34). Much more work has been done on the histopathology of type A, and the following lesions are noted:

a. heart--cloudy swelling of the fibers, occasional hyaline degeneration, petechial hemorrhages beneath the covering of serous membranes, occasional degeneration and necrosis with slight calcareous infiltration in the Purkinji cells, and hemorrhages in the epicardium, coronary furrow and subendocardium.

b. kidney--extensive degeneration and necrosis of convoluted tubules, precipitation in the lumen of the tubules, subcapsular hemorrhages, congestion and hemorrhage in the medulla, and a friable parenchyma.
c. spleen--petechial hemorrhages throughout and pulpy in consistency.
d. lung--slight to marked congestion, serous exudate in the alveoli.
e. liver--cloudy swelling and karyolysis of some small groups of cells.
f. lymph nodes--edema and peripheral congestion with slight hemorrhage (32).

No agreement can be found among authors as to the age at which type A occurs. Macrae (25) reports that it occurs in the first week of life, but Schofield (32) states it occurs between 6-10 weeks, and Shirley (34) claims that it is seen only in older cows.

Type B. According to Hepple (23), in calves a severe diarrhea fatal in 1-4 days is caused by type B. Small yellowish diphtheritic patches distributed in necrotic areas along with congestion of the mucous membrane of the caecum and colon (23) are the major differences noted. The liver, spleen and kidneys are intensely congested with blood. The lungs are reported to be normal (23).

Histopathology of the mesenteric lymph nodes shows an active vascular reaction with hyperemia of venulae and cellular infiltration. The kidneys show subcapsular hemorrhage, swelling of the convoluted tubules, areas of congestion in the medulla and some nuclear fragmentation (23).

Type D. Enterotoxemia caused by type D is reported in young calves by Griner, Aichelman, and Brown (12), and Schofield (31), and in cows by Keast and McBarron (24). It is characterized by central nervous symptoms, convulsions, incoordination, blindness, opisthotonus and head pushing (12). A severe pulmonary edema and diffuse red specking of the trachae with much froth in the air passages is seen (6, 24). Considerable gas was also reported to be present in the caecum and colon by Keast and McBarron (24). Griner and associates (12), along with Keast and McBarron (24), agree that the condition closely resembles acute D enterotoxemia of sheep.

Type E. The diarrhea present in type E enterotoxemia ranges from yellow to orange mucus. All other symptoms reported by Griner were similar to type C (12).

To quote Griner, "The symptoms of enterotoxemia caused by the 6 types of Clostridium perfringens in various

species are in general similar to those caused by type D in lambs (12)."

Recent findings by Griner (13) in acute and subacute forms of Clostridium perfringens type D enterotoxemia in lambs are of value to this study. The histopathology of the brains of dying lambs is very similar in location to that of animals with the subacute form. Microscopic foci of softening or liquefaction necrosis are formed in the basal ganglia, thalamus, internal capsule, substantia nigra, subcortical white matter, and cerebellum of affected lambs. The lesions are characterized by vascular congestion, degeneration of endothelium and walls of the vessels, pronounced perivascular edema and varying degrees of intercellular edema. Pathologic changes in the neurons and neuroglia appear to be related to the increase in plasma transudate (13). The use of radioactive I^{131} revealed a sharp increase in the distribution of the isotope in the brains of lambs intoxicated with Clostridium perfringens type D toxin, thus indicating a marked increase in vascular permeability (19). Also, the chronologic pathogenesis of encephalic lesions in type D intoxicated mice appeared to be related to an initial increase in vascular permeability followed by edema, softening, liquefaction necrosis, and healing by glial scarring (13). It therefore appears that the primary action of Clostridium perfringens type D toxin is on the vascular system, causing an increase in permeability (19). These

findings are in agreement with the findings of Bullen and Batty (8), who reported that oral administrations of type D toxin increased the permeability of the mouse intestine. No gross lesions were reported to occur by either author.

Diagnosis:

Due to the rapid course of enterotoxemia, symptoms are frequently missed (12). A presumptive diagnosis of hemorrhagic enterotoxemia can be made when young suckling calves are found dead, and autopsy shows extensive hemorrhagic enteritis (1). The classic method of identifying individual species of bacteria by the morphological, cultural, physiological and pathogenic features is helpful and should be a standard procedure in the laboratory; however, it doesn't definitely type the toxin (28).

Field diagnosis can be accomplished by the use of specific type antitoxin or vaccine. By injecting part of the subjects with type A, type B, type C, or type D, respectively, and leaving part to serve as a control, one is able to determine the causative organism. However, this method is very impractical (12).

Confirmation of a diagnosis can be obtained by sending 25-50 cc. of intestinal contents to a diagnostic laboratory (15). Here, the presence of gram positive, nonmotile, medium sized bacilli morphologically resembling Clostridium perfringens seen in a direct smear of intestinal contents

is considered helpful (17, 26). Upon anaerobic incubation on blood agar, a hemolytic zone around each colony should be produced. A stormy fermentation is shown in milk (17).

Typing of the toxin is carried out in the laboratory utilizing various tests. Perhaps the most practical is by toxin neutralization with specific antitoxin or sera. Sera has antitoxin in it for neutralizing toxins produced by various species in the intestine or the media of the laboratory. Each serum is type specific and may have 1 to 6 recognized antitoxins in it. All of the 6 known types of Clostridium perfringens can be identified by recognizing the toxin or combination of toxins produced (41). The antitoxin sera are prepared in hyperimmune horses and international standards have been established for each serum (37).

Routine laboratory diagnosis is performed on the supernatant fluid of the intestine (9), or more preferably on the bacteria-free filtrates of the intestinal contents (15). The intestinal contents are diluted with equal parts of distilled water and filtered through a seitz filter. The filtrate is then inoculated into mice, 0.3 cc. (41) intravenously into the lateral tail vein. If toxin is present, death will occur in 30 minutes to 3 hours (17). If the filtrate is lethal, prepare mixtures of 0.9 cc. of test fluid plus 0.3 cc. of the different types of sera A, B, C, and D. Incubate the mixtures for 1/2 hour at room temperature and inject 0.4 cc. (41) or 0.3 cc. (17) of each

mixture intravenously into mice along with 0.3 cc. of the test fluid as a control. The results are read up to 3 days (41).

A simple typing chart adapted from Frank (9) is as follows:

Typing of Clostridium perfringens.

Filtrate Neutralized by Serum type	Filtrate Not neutralized by Serum type	Interpretation of results Toxin	Type
A	--	A	A
B	A, C, D	B, E	B
C	A, D	B	C
D	A, C	E	D
Bosworth	A, B, C, D	Lambda	E

Type D toxin is neutralized by its own antitoxin and type B antitoxin, but not A or C antitoxin (37). Type C antitoxin will neutralize the filtrate of a young culture of type B, because at this time the beta, gamma, and delta toxins are formed while epsilon protoxin is in its nontoxic state. After time, however, the epsilon protoxin becomes activated, and type C antitoxin will no longer neutralize type B toxin. Intravenously, the beta toxin causes an increase in respiratory rate, followed closely by chronic nervous spasms, hind limb extension and death. Therefore, it should be kept in mind that the beta toxin will usually kill mice within minutes after injection (37).

Stableforth and Galloway (37) consider intracutaneous tests in guinea pigs more sensitive than mouse intravenous tests. The beta toxin gives a purplish congested area which eventually becomes necrosed. The lesion is irregular in shape and spreads due to the presence of hyaluronidase (37). Rabbits and mice may also be used for the intracutaneous tests (41).

It may be assumed that when beta toxin is demonstrated, the causative organism is either type B or C. Likewise, if epsilon toxin is observed, B or D could be the causative organism (9). Beta toxin is in highest concentration in early cultures and is readily inactivated by trypsin (28). Therefore, using the methods of Bosworth and Glover (1934), epsilon toxin of Clostridium perfringens can be demonstrated by activating the filtrate with trypsin and retyping again in mice to differentiate between types B and C (23). In the case of culture filtrates that are typed B, they should be rechecked in 72 hours for the presence of epsilon toxin (23).

Montgomerie clearly states in his work that he was never able to demonstrate the presence of both beta and epsilon toxin in a single sample of intestinal contents (27). This was confirmed by Frank (9). However, Frank states he has found flocks of sheep with both types C and D occurring in them (9). Toxin neutralization tests can also be used to measure antitoxin unitage of animal titer (17).

Some laboratories prefer to grow the organism in 1% glucose broth, modified Brewer's media or similar medium. It is filtered through Seitz sterilizing pads before being examined for toxins (30, 17). The filtered toxins are then tested by toxin neutralization tests and for haemolysin, lecithinase and collagenase (30, 37, 41). See Table I, page 4, for results and Oakley and Warrack (30) for procedures.

Barner states that the diseases to be considered in making a differential diagnosis are the following: (1) hemorrhagic septicemia; (2) coccidiosis; (3) listeriosis; (4) plant or chemical poisoning; (5) botulism; (6) white muscle disease (2).

Griner reports that a positive diagnosis of type C enterotoxemia can be made if the following three conditions are met:

1. Smears of intestinal contents show organisms resembling Clostridium perfringens.
2. Types B and C antitoxin neutralize the toxin.
3. Type C antisera prevents the condition (20).

Treatment:

Few diseases or conditions of domestic animals demonstrate the value of preventative medicine as does enterotoxemia (12). In many cases, enterotoxemia is observed in animals too young to be actively immunized by vaccine, so

control must take on the form of passive immunity by: (1) administration of hyperimmune antitoxin serum as soon after birth as possible; (2) ingestion of colostrum milk from a dam previously immunized with toxoid (17, 26, 39). Older animals can be protected either actively or passively by using antitoxin sera, a vaccination with highly antigenic toxoids, or bacterins (12, 26).

Antitoxin is derived from horses which have been hyperimmunized against the type of toxin desired (39). Prophylactic dosage is 10 cc./calf subcutaneously. The antitoxin titer lasts approximately 3 weeks in the calf (1, 17, 39, 40). Due to the similarity in symptoms of enterotoxemia, which is caused by the various types of Clostridium perfringens, it is recommended that in actual cases the practitioner use the combination antitoxin B.C.D. rather than risking greater losses (3, 22, 42). It should be noted that Clostridium perfringens type B.C.D. antitoxin is actually a combination of type C and D antitoxin and does not contain 3 major fractions or antitoxins as the name implies (1).

Therapeutically, antitoxin can be of some help in the early stages of the condition in dosages of 25-30 cc. intravenously or subcutaneously (1, 39, 40). The above dosages are based on the products having a minimum of 1500 units of a specific antitoxin/cc. (1, 39). It must be kept in mind that when using hyperimmune serum, one is injecting

a foreign protein; therefore, precautions should be taken to handle any foreign protein or anaphylactic reaction (1, 3).

To provide passive immunity to the offspring, the dam is usually vaccinated 2-4 months prior to calving with a booster injection given 3 weeks after the initial injection. Griner states, however, that ranchers object to the 2 injections being given within a 3-week period (12). A single booster dose is recommended for the second year and years following. The recommended dosage is 5 cc. in cows with 30 toxoid units/cc. being the minimum standard for the vaccine (11, 17, 22, 26, 39, 40). Initial injections will give a titer in 3 weeks (12), and the second injection provides a higher response in 7 days (39).

Toxoids give much better results than bacterins (39). They can be used in younger animals and produce a higher longer lasting titer. Lambs as young as 15 days have produced immunity (12, 22). Bacterins cannot be used in animals under 8 weeks of age. If used in older animals, the resulting immunity is lower and short lived (22).

The toxoid product is activated with trypsin and then detoxified to produce the vaccine. Toxoid immunity comes from a sensitization to the antigen. The second dose of toxoid has a booster effect by sensitizing the animal to the specific toxin. Upon contact with the specific toxin, the animal immediately responds with a rapid production of antitoxin (22, 39, 42). A small percentage of animals fails

to respond and never does develop an antitoxin titer (42).

The object of vaccinating close to the end of pregnancy is to obtain as high an antitoxin titer as possible at the time of calving. The higher the blood antitoxin of the dam, the higher the colostrum antitoxin and the greater the protection to the calf (1). The effectiveness of active immunizing agents against the organism is debated and difficult to determine because of the unpredictability of natural occurring cases (35).

Griner and Baldwin found in their work that antitoxin may be cumulative in calves, since, in many cases, the antitoxin titer of the calf exceeded that of the dam (1, 17). Griner also found that 14% of the cows and 24% of the calves he tested had natural occurring titers (17).

Prophylactic chemotherapy, in the form of feed additives, has been used and is currently being evaluated. McGowan reported significant results with chlortetracycline, as a feed additive, in the control of enterotoxemia in lambs (12). Griner reports in another article that antibiotics are of value when used prophylactically, but their value as therapeutic agents is doubtful, as they are not capable of neutralizing the lethal toxin (15). In Hagan and Bruner, it states that the addition of sulfur to the ration to restrict the intake of feed, has been successful in reducing enterotoxemia (21).

In the Annual Review of Microbiology (43), Williams

showed that oral and cloacal administration of Clostridium perfringens had no effect on the growth of the chickens. He further stated that his findings failed to support the hypothesis that Clostridia in the feces elaborate toxins which inhibit growth, and that the growth-stimulating effect of antibiotics in the diet, is due to the suppression of these Clostridia (43).

PART III

MATERIALS AND METHODS

The *Clostridium perfringens* type C (beta) toxin used for this experimental work was obtained from the research division of a veterinary pharmaceutical company.* The toxin was sent air express in three shipments, February 9, June 17, and July 20, 1961. It was packed in dry ice and arrived in a semi-frozen state. Upon arrival, it was allowed to liquefy at room temperature, and was then transferred to 1 cc. and 5 cc. vials, refrozen immediately, and stored at -5 F. until used. The toxin was kept frozen to avoid a loss in potency and when needed, it was liquefied at room temperature and used promptly.

Accompanying the toxin was the following description on how it was obtained:**

> "In my previous work in screening organisms to obtain this beta toxin, found one of the 'American type culture collection' #3626 *Clostridium perfringens* Type agni to give the best results in my hands.
>
> Media:
>
> A. Take 5 pounds fresh ground beef liver, cover

---

*Haver-Lockhart Laboratories, Kansas City, Missouri, Dr. F. W. Binkley, research Clinician. Toxin was originally sent to Dr. G. R. Moore, Director of Large Animal Clinics, Michigan State University.

**Quoted directly from Dr. F. W. Binkley's description.

with distilled water; boil for thirty minutes and press.

B. Heat broth and add 1% peptone (50 Gm.), 0.1% dibasic potassium phosphate (5 Gm.), 0.02% crystine (1.25 Gm.).

C. Adjust to pH 8.2.

D. Boil for 3 minutes and filter (coarse).

E. Make up to 5,000 cc. with distilled water and fill into flasks (300 cc.).

F. Fill deep anaerobic tubes containing small quantity of pressed liver (use these for seed tubes).

G. Sterilize for 45 minutes at 15 pounds pressure. The seed is carried in (F) above and three quick transfers are made--Monday, 8:00 A.M.; Tuesday, 8:00 A.M.; Tuesday, 4:00 P.M.--then inoculate flask (E) with one tube of (F) Wednesday 8:00 A.M. Harvest 2:00 P.M. (Wednesday) or 6 hours growth. The next step is to centrifuge to pull out cells and lower particulate, then filter through ST -3 Hercules filter pad to remove cells and leave pure filtrate of toxin. In looking up the habits of this organism, you will find that the Beta toxin comes off first, between 4 and 8 hours; between 8 and 12 hours, the Theta toxin comes off and later, the Epsilon toxin. The A toxin comes off in unknown quantity anytime. However, your toxin has proven to be almost completely pure Beta. Beta toxin here should titer out to be between 800 and 1000 MLD/cc. for mice.

Previous experience indicates that 1 cc. of this i.v. to a ewe will kill in 15 minutes if she had no protective antibodies."

Prior to experimentation, the toxin was typed, using sera obtained from Wellcome Research Laboratories, London, England. The sera, which are type specific, contain the antitoxins for neutralizing the toxins produced by the various species of Clostridium perfringens organisms. The

method of typing was in accordance with directions accompanying the toxin as follows: Three white mice (17-20 Gm.) were injected intravenously with 0.3 cc. of test fluid (toxin) and, if this proved lethal, mixtures were prepared using 0.9 cc. of test fluid and 0.3 cc. of the different type sera A, B, C, D, Bosworth. The mixtures were allowed to stand at room temperature for half an hour; then 0.3 cc. of each mixture A, B, C, D, Bosworth and, as a control, 0.3 cc. of test fluid, was injected into white 17-20 Gm. mice. Four mice were used for each mixture. The results were read up to three days and the interpretation was based on a chart similar to the table on page 30.

The minimal lethal dose (MLD) of each shipment of toxin was determined by intravenous inoculation into white 17-20 Gm. mice. After thawing at room temperature, the toxin was agitated and 0.1 cc. was pipetted into a 10 cc. volumetric flask which was then filled to volume with .85% sodium chloride solution (saline) to make a 1-100 dilution. Serial dilutions were made from the 1-100 dilution by taking 1 cc. and diluting with the proper amount of saline to make the desired dilution. A preliminary screening of the toxin was done at dilutions of 1-100, 1-200, 1-300, 1-400 on three white mice per dilution. After estimating the MLD, five dilutions were prepared--one at, two above, and two below the estimated endpoint--each differing by a dilution factor of 25. The diluted toxin was then injected intravenously

into 10 mice for each dilution. The results were read between 5 minutes and 4 hours and the MLD 50 was figured for mice.

Trypsinated toxin was prepared by mixing 0.1 cc. of 0.25% trypsin solution (pH of 8 or higher) to 1 cc. of toxin. The mixture was incubated for one hour at 37 C. and injected.

White mice* weighing between 17 and 20 grams were used for titrating the toxin, checking the sera and other procedures conducted in this research project. Before injection, the mice were placed in a pail and warmed with a 100 watt light bulb to cause engorgement in the coccygeal veins. Care was taken not to overheat the mice. Intravenous injections were made into the lateral coccygeal vein using a 0.5 cc. tuberculin syringe equipped with a 1 inch, 27 gauge needle. For restraint, they were placed in a holder made from a large centrifuge tube. Before injecting intravenously, the tails were wiped with 50% alcohol. After injection, mice were placed in a clean, dry cage until the results were recorded. In all cases, results were taken between 5 minutes and 4 hours except in the case of typing, where the time lapse was up to 3 days.

For this study, 15 clinically normal steer calves

---

*Obtained from Rawley Farms, Plymouth, Michigan, and Spartan Research Animals, Okemos, Michigan. Seven hundred twenty-five mice were used in this problem.

were obtained from the Michigan State University dairy farm. Seven of the calves were Holsteins, three Brown Swiss, three Jerseys, and two Guernseys. They varied in age from 1 week to 11 months and in weight from 60 to 345 pounds. All the calves were housed in the basement of the large animal clinic.

Serum from each calf was checked for Clostridium perfringens antitoxin prior to use in this experimental work. Ten cc. samples of jugular vein blood were centrifuged at 1350 rpm. for two minutes and the serum removed. Then 1 cc. of the toxin was diluted to contain 12 MLD/cc. One cc. of the diluted toxin (12 MLD/cc.) was incubated with 1 cc. of the serum at 37 C. for 1 hour. Following incubation, 0.3 cc. of this mixture was injected intravenously into each of three mice. As a control, 1 cc. of 12 MLD/cc. toxin was incubated with 1 cc. of normal saline solution and 0.3 cc. of the resulting mixture was also injected into each of three mice. Death of the mice indicated that the calf did not have any protective antibodies at this level. If the mice lived, the calf was not used, since it presumably had antitoxin against the Clostridium perfringens toxin.

After determining that the sera of the calves did not contain antitoxin and much preliminary work, six calves were given intravenous doses of toxin at the rate of 15,000 mouse MLD's per 100 pounds of body weight.

Prior to injecting the Clostridium perfringens toxin into the calves, rectal temperatures, heart rates, respiratory

rates, blood and urine samples were taken for control data.

Control urine samples were collected by stimulating the steer to urinate by massaging the prepuce with warm water. A plastic bag was then suspended under the prepucial orifice with adhesive tape to collect urine samples as they were voided. The urine was injected intravenously into mice in 0.1 cc. and 0.2 cc. quantities to determine the presence of a lethal factor.

Samples of jugular vein blood were collected in vials containing ammonium-potassium oxalate crystals prior to and 0.5, 3, and 12 hours post injection. The blood was used for total and differential leukocyte counts and packed cell volume determinations (micro-hematocrit). All blood studies were done according to the Manual of Clinical Laboratory Methods.*

To test for the presence of toxin in the calves' blood, 10 cc. samples were drawn from the left jugular vein prior to and at 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, and 60 minutes post injection. Later in the experiment, ammonium-potassium oxalate was used as an anticoagulant to eliminate the formation of fibrin clots in the serum. The samples were immediately taken to the laboratory and centrifuged at 1350 rpm. for two minutes and the serum removed. The serum (0.2 cc.) was then injected intravenously into 17-20

---

*O. E. Hepler, Charles C. Thomas Publisher, Springfield, Illinois.

Gm. white mice, three for each sample. This was done to determine how long an MLD of toxin for a mouse remained in the blood stream of the calf.

The five goats used in this project were of the Toggenburg breed and varied in weight from 140 to 160 pounds. They were also housed in the basement of the large animal clinic.

PART IV

RESULTS AND DISCUSSION

Typing of toxin:

Three days post injection of the toxin incubated with the different types of sera, the following results were observed.

TABLE I. Typing of toxin[1].

	Inoculum used	Mouse Inoculated			
		1	2	3	4
Control	Test fluid + saline	+	+	+	+
A	Test fluid + Sera A	+	+	+	+
B	Test fluid + Sera B	0	0	+	0
C	Test fluid + Sera C	0	0	0	0
D	Test fluid + Sera D	+	+	+	+
"Bos-worth"	Test fluid + Sera E	+	+	+	+

0 = mouse lived
+ = mouse died within 3 days
1. for procedure, see page 39 of Materials and Methods. 0.9 cc. of test fluid was combined with 0.3 cc. of sera incubated and 0.3 cc. of this mixture was inoculated intravenously into the lateral coccygeal vein of mice.

From the above table, it is evident that the toxin received from Haver-Lockhart Laboratories was neutralized

by sera type B and C and not by A, D, or Bosworth. In checking the table in Review of Literature, page 7, it is noted that beta toxin is the major toxin in both Clostridium perfringens type B and C. Applying the above information to Frank's table given in Review of Literature, page 30, it becomes evident that we are dealing with beta toxin.

Sera Antitoxin test:

The serum of each calf was checked for Clostridium perfringens type C antitoxin (see page 41) before the calf was used for experimental work.

All calves were negative before the initial administration of the toxin.

It was found that 0.3 cc. of serum injected intravenously killed mice in 8 out of 10 cases. This rate of death due to serum could be greatly reduced by diluting 0.2 cc. of serum with 0.1 cc. of saline. By diluting the serum, the death rate was reduced to one death out of ten inoculations. In the serum antitoxin test, 1 cc. of serum was diluted with 1 cc. of toxin, thus reducing the toxic effect of the serum.

The incompatibility of mouse blood and cattle serum was further examined by plate agglutination observations. Sera from six calves, when matched with blood of different mice, caused agglutination of the red blood cells in degrees varying from slight to severe.

TABLE II. Test for serum antitoxin[1].

Before Administration of Toxin					After Administration of Toxin			
Calf No.	Mouse Number 1	2	3	Presence of antitoxin in Calf Serum	Mouse Number 1	2	3	Presence of antitoxin in Calf Serum
10	+	+	+	Negative	0	ϴ	ϴ	Positive
11	+	0	+	Negative	0	0	ϴ	Positive
14	+	+	+	Negative	Not rechecked			
21	+	+	+	Negative	Not rechecked			
22	ϴ	+	+	Negative	ϴ	ϴ	ϴ	Positive (?)
23	0	+	+	Negative	0	0	0	Positive
25	+	+	+	Negative	Not rechecked			
26	+	+	+	Negative	Not rechecked			
27	+	ϴ	+	Negative	Not rechecked			
28	+	+	+	Negative	Not rechecked			
30	+	+	0	Negative	ϴ	+	ϴ	Positive
31	+	+	+	Negative	+	+	ϴ	Negative
34	+	+	ϴ	Negative	Not rechecked			
42	+	+	+	Negative	Not rechecked			
45	0	+	+	Negative	0	0	0	Positive
Control toxin + saline	+	+	+					

0 = mouse lived
+ = mouse died
ϴ = mouse sick

1. Serum (1 cc.) from calves was incubated with 1 cc. of diluted toxin (12 MLD/cc.) and injected intravenously into mice.

Upon checking the serum of the calves after exposure to Clostridium perfringens toxin, an antitoxin response was noted in all but one animal. The response varied and was not constant, probably due to the different routes of administration and different levels of toxin used. No attempt was made to measure how much antitoxin was formed by the animal. Part of the calves were not rechecked due to their loss in non-related experiments or to termination of the project.

Minimal lethal dose of toxin:

Dr. F. W. Brinkly of Haver-Lockhart determined the toxin to be between 800-1,000 MLD/cc. Since the last two shipments, June and July, 1961, were harvested at the same time from the same culture, it was necessary to standardize the toxin only twice--once for the March shipment and again for the June and July shipments.

It was decided to use LD_{50} to determine the potency of the toxin and then to use 1/2 the dilution factor for LD_{50} to get the LD_{100}. It was felt this procedure would prove more accurate and give a sharper endpoint.

The LD_{50} was defined as the dose of toxin required to kill 50% of 17-20 Gm. white mice injected intravenously. The results were read between five minutes and four hours.

The procedure was carried out according to the methods listed in Materials and Methods, pages 39-40, and the results are tabulated below:

TABLE III. Calculation of LD_{50} for Clostridium perfringens Type C Toxin

Toxin received in March				Toxin received in June			
Dose 0.3 cc. of a:	Results Lived	Died	Mortality Percentage	Dose 0.3 cc. of a:	Results Lived	Died	Mortality Percentage
1/250 dil.	0	10	100%	1/125 dil.	0	10	100%
1/275 dil.	2	8	80%	1/150 dil.	3	7	70%
1/300 dil.	4	6	60%	1/175 dil.	4	6	60%
1/325 dil.	6	4	40%	1/200 dil.	6	4	40%
1/350 dil.	9	1	10%	1/225 dil.	8	2	20%

LD_{50} dil. = 1/319*

Mouse MLD_{100}/cc. = $\frac{1/319}{2}$ x 3.3 = 528/cc.

LD_{50} dil. = 1/182*

Mouse MLD_{100}/cc. = $\frac{1/182}{2}$ x 3.3 = 300/cc.

Throughout the experiment all toxin was adjusted to contain 500 mouse MLD per cc.

Type C toxin:

Preliminary work:

On May 1, 1961, 1 cc. (500 MLD) of Clostridium perfringens type C toxin was injected intravenously into the right jugular vein of a one week old, sixty pound Jersey

*The above figures were arrived at by applying the information in Table III to the method for figuring LD_{50}, as given on page 39.

calf. The only reactions observed were a slight lacrimation from both eyes and two short dry coughs. One hour later, 2 cc. of toxin were injected into the left jugular vein of the same calf; within two minutes, the calf coughed, showed lacrimation in both eyes, showed colic symptoms, and urinated. Five minutes post injection, the calf was seemingly normal and remained so.

The above results were also observed when the toxin was given to another Jersey calf and to two Guernsey calves. The dose varied from 5 cc. intravenously for the Jersey, to 10 cc. for an eighty-pound Guernsey, and 20 cc. for a 105-pound, three week old Guernsey. No increase in severity of symptoms was noted with the increasing dosage, and all of the animals lived without showing any ill effects.

In goats:

The toxin was then tried intravenously (I.V.) in five goats, the dose varying from 0.75 cc. to 1.5 cc. The goats all died within four hours of injection; two within fifteen minutes. The symptoms noted were frequent bleating, grunting, colic, running movements, bloating, respiratory distress and opisthotonous.

Post mortem examinations were performed on three of the five goats, and the following conditions were observed: fluid in the thoracic cavity; excessive edema (white frothy) in the lungs, tracheae and nostrils; pericardial

edema; hemorrhages on the endocardium; bloat and gas in the large and small intestines; a few petechial hemorrhages were noted on the meninges and on the cortical surface of the brain. One of the goats showed excessive fluid in the spinal canal.

Trypsinated toxin and trypsin:

The literature suggested the use of trypsin as a trigger mechanism. A 95-pound, three month old Jersey calf was given 5 cc. of trypsinated toxin intravenously. Two minutes following injection, the calf coughed, urinated, defecated, showed colic, and severe respiratory distress. The respiratory symptoms persisted, and 30 minutes post injection, the calf showed disturbances in the central nervous system and lost control of its righting reflex. The central nervous system and respiratory symptoms progressed, until the time of death, two hours and forty minutes post injection. The calf died in opisthotonous with white foam coming from the nostrils while showing running movements.

Upon post mortem examination, there was excessive fluid in the spinal canal and hemorrhages were present in the brain. Hemorrhages were also present on the heart and pericardium. Also observed were emphysema of the lungs, and the trachea and bronchi were filled with a white, foamy material.

Trypsin (5 cc.) was then injected intravenously

into a 140-pound Jersey calf and within one minute, the calf was down, gasping for breath, with legs apart. The animal was unable to rise and gave no reaction to various stimuli. One hour later, the animal was up and appeared normal.

Trypsin was then diluted with normal saline to form 50% (1 cc. trypsin ⁄ 1 cc. saline), and 10% (5 cc. trypsin ⁄ 45 cc. saline) solutions. The solutions were injected intravenously into two different Holstein calves weighing approximately 250 pounds each. The symptoms observed following injections were as follows: shallow moist cough, respiratory distress, lacrimation from both eyes, excessive salivation, abdominal contractions, and extension of the neck. Both animals were normal and eating within one hour.

Trypsin and trypsinated toxin, in the above dilutions, were also given intravenously to thirty-six mice. Death was produced in thirty-two.

Two cc. of trypsinated toxin which was given intravenously to a 140-pound goat produced death within fifteen minutes.

Orally:

Pure toxin was administered orally in milk at 5 cc., 10 cc., 15 cc., 20 cc., and 30 cc. dosage levels to five different calves, varying in weight from 75 to 100 pounds. One calf that received 10 cc. died. This calf was being

taught to drink from a pail, apparently without much success, because obvious malnutrition was present. The other animals remained normal.

Intraduodenally:

A laparotomy was performed on the right side of a 120-pound, three week old Brown Swiss calf, and 20 cc. of toxin were injected into the duodenum. No reaction to the toxin was observed. The sera of this animal checked out negative for antitoxin before and after injection.

Intravenously:

After injecting 20 cc. of toxin into the right jugular vein of a 250-pound Holstein calf, blood samples were taken from the left jugular vein at one, five, ten, and thirty minute intervals. The sera was removed, and 0.3 cc. of sera was injected intravenously into mice to test for toxin. It was found during this procedure, that by cross matching and using a non-injected calf's sera, that mice were killed by sera if given quickly or in 0.3 cc. quantities without dilution.

From twenty mice injections, it was determined that sera could be given in 0.1 cc. or 0.2 cc. doses without harming the mice; thus, it would be possible to test for the presence of toxin in the calves' blood.

Six steer calves (code numbers 14, 21, 25, 26, 27,

and 28), whose sera checked negative for antitoxin, were chosen to study the effect of 15,000 mouse MLD (30 cc.) of toxin per 100 pounds body weight of calf. The calves varied in weight from 131-345 pounds, and the dose of toxin injected intravenously in the right jugular vein ranged from 39.3-103.4 cc.

Temperature reaction:

The rectal temperature of the six calves rose one degree, on the average, within five minutes post injection of the toxin, and then, within thirty minutes, dropped to the temperature recorded prior to injection. (See Chart I.) After three hours, the rectal temperatures were below those taken before experimentation. The fact that pre-injection temperatures were slightly elevated may be explained by excitement in preparation for the experiment.

Heart and respiratory rate:

Within two minutes after toxin injection, the heart and respiratory rates were doubled in all but one calf. These gradually returned to normal as the experiment progressed. (See Charts II, III.) Although in the one calf in which the rates did not double, there was still a considerable increase. No explanation can be given for the slight depression of heart rate between the ten and fifteen minute post injection period.

CHART I. Body Temperature Reaction to Toxin Administration

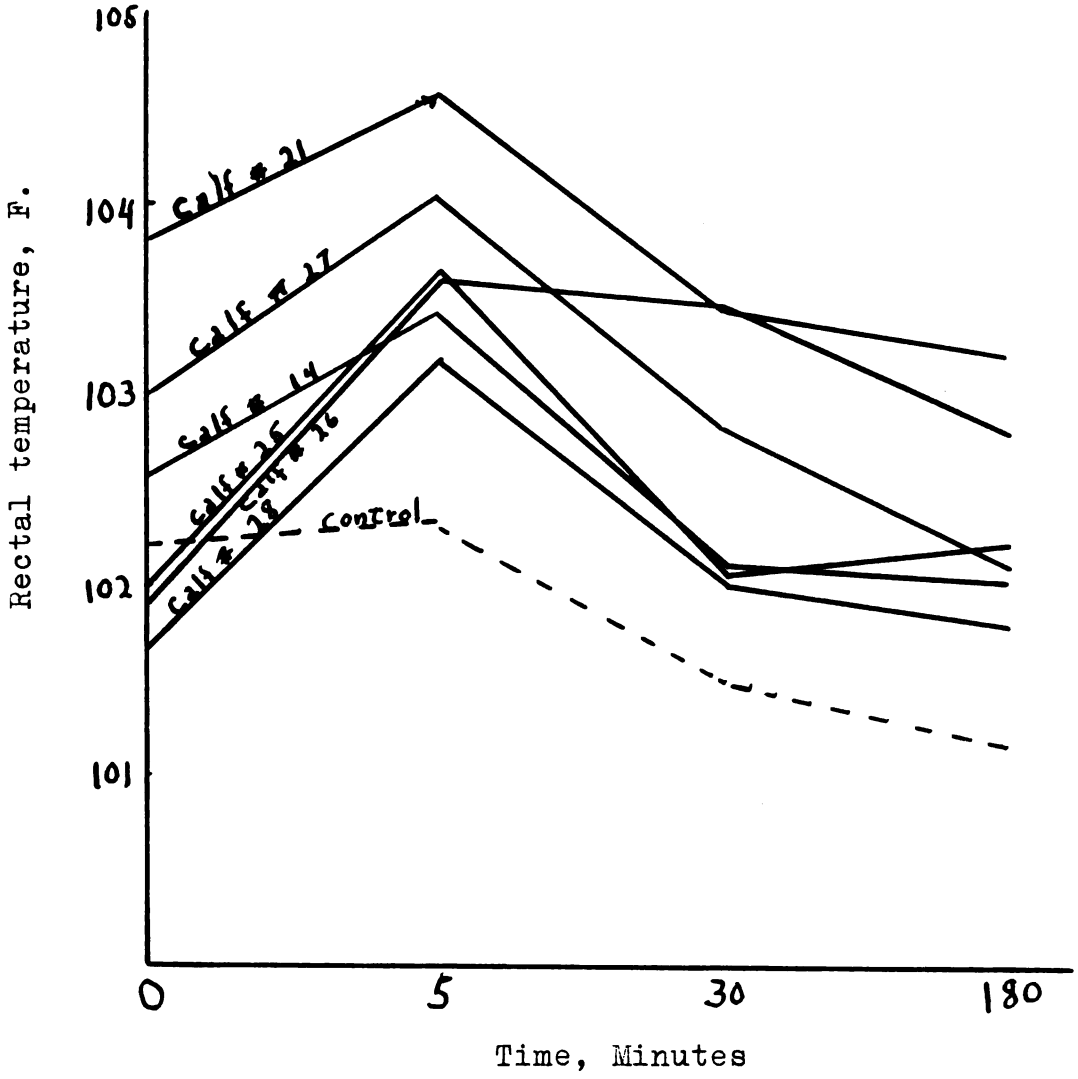


CHART II. Respiratory Rate Following Toxin Administration

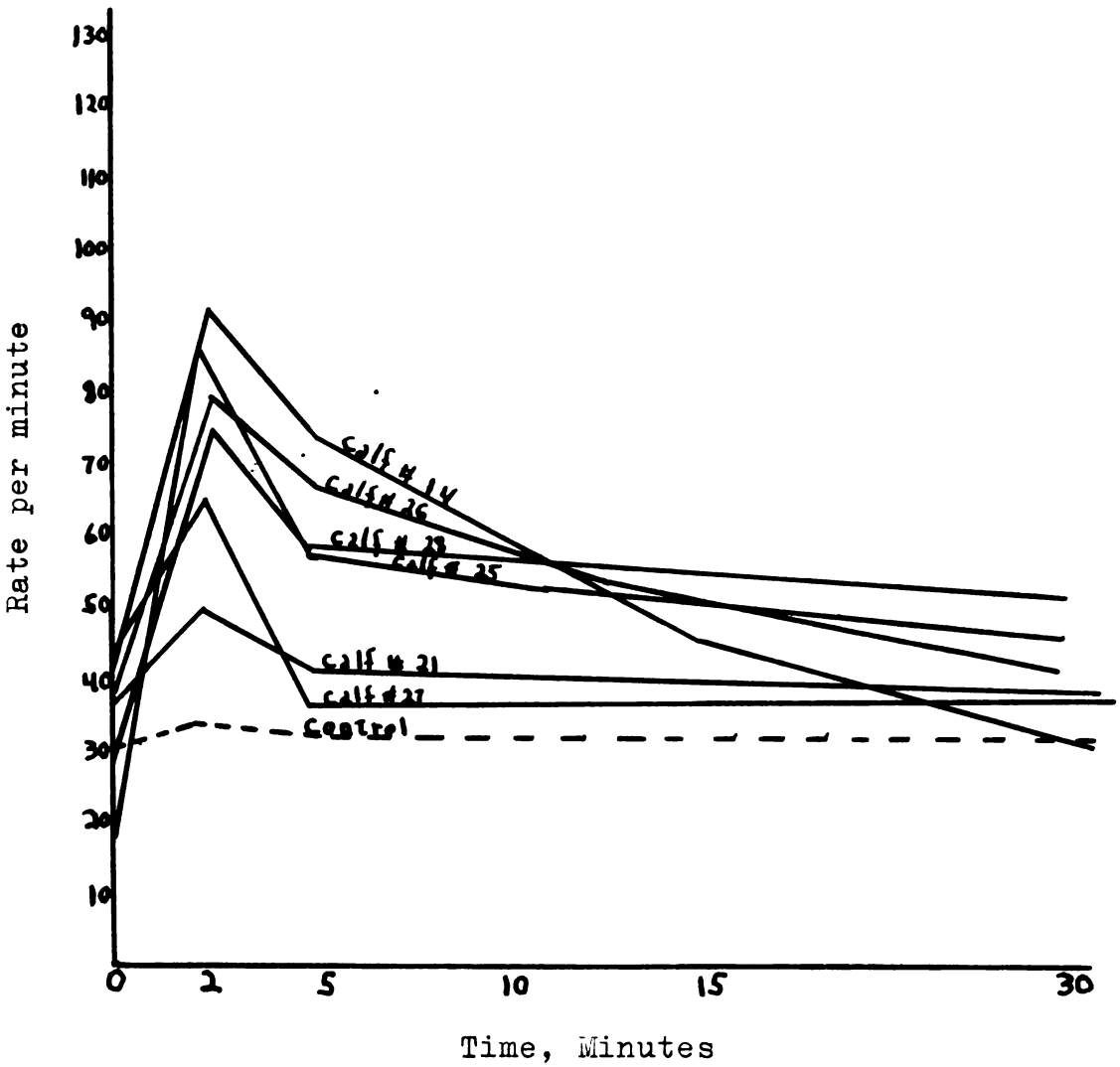
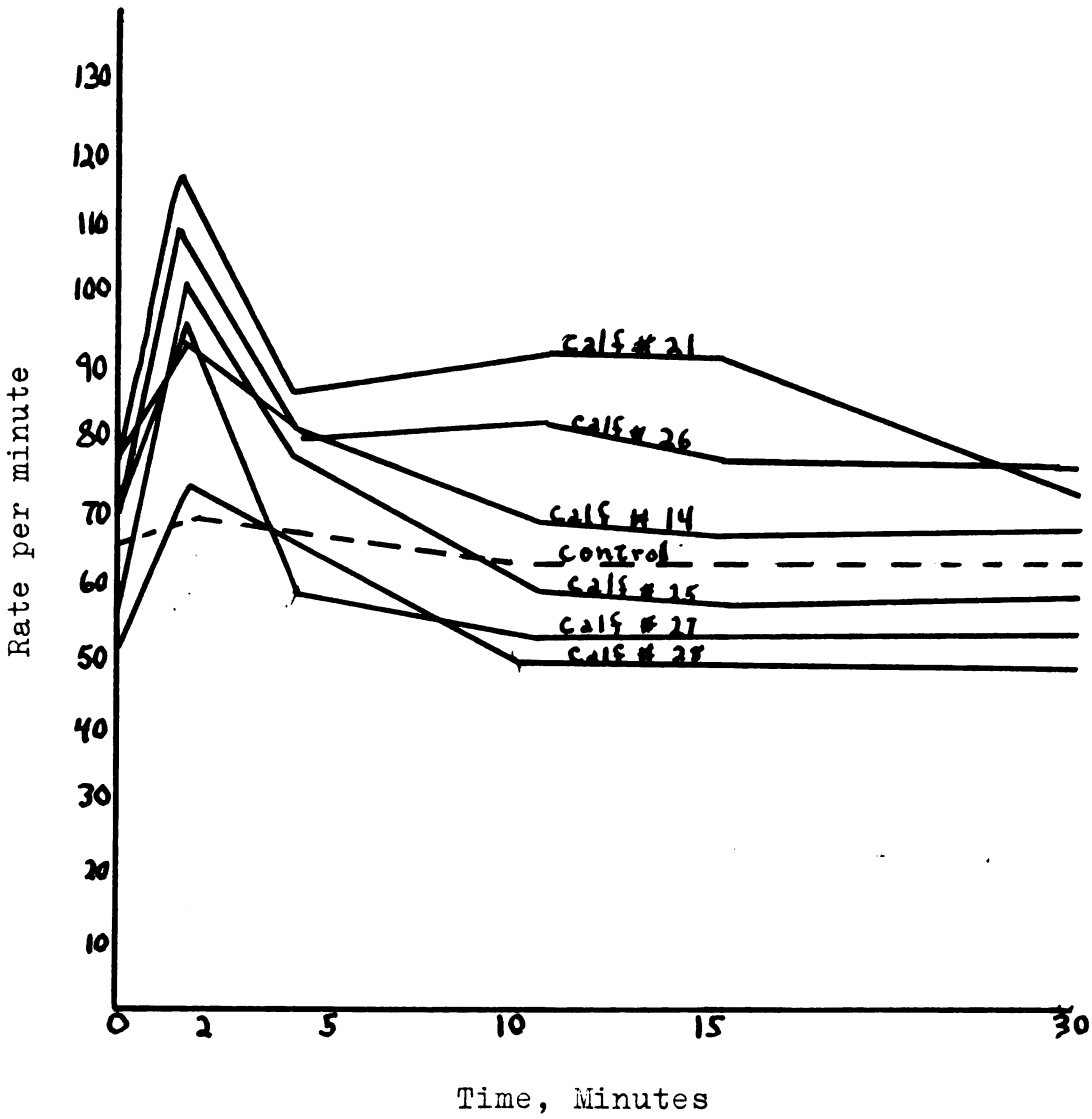


CHART III. Heart Rate Following Toxin Administration



Persistence of toxin in the blood:

Blood samples from the left jugular vein were collected at intervals as shown on Table IV. Sera from these samples were injected into mice in 0.1 cc. and 0.2 cc. quantities to check for the persistence of the toxin. Results were read between five minutes and four hours on whether the mice lived, died, or became sick.

TABLE IV. Persistence of intravenously injected toxin in calves' sera[1].

Calf Number	14		21		25		26		27		28	
Quantity[2]	.1	.2	.1	.2	.1	.2	.1	.2	.1	.2	.1	.2
Sample Time[3]												
Prior	00	00	00	00	00	00	00	00	00	00	00	00
2 min.	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
4 min.	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx	xx
6 min.	x~~0~~	xx	~~0~~x	x~~0~~	xx	xx	xx	xx	~~0~~x	xx	x~~0~~	xx
8 min.	~~0~~x	xx	~~0~~0	~~0~~~~0~~	~~0~~x	xx	xx	xx	~~0~~~~0~~	~~0~~x	x0	xx
10 min.	0~~0~~	xx	00	0~~0~~	~~0~~0	~~0~~x	0~~0~~	xx	00	0x	0~~0~~	xx
15 min.	00	~~0~~~~0~~	00	00	00	~~0~~0	x0	xx	00	~~0~~0	00	~~0~~x
20 min.	00	~~0~~0	00	00	00	00	00	xx	00	00	00	~~0~~~~0~~
25 min.	00	00	00	00	00	00	00	~~0~~x	00	00	00	~~0~~0
30 min.	00	00	00	00	00	00	00	00	00	00	00	00

0 = live mice
~~0~~ = sick mice
x = dead mice

1. mice were used as the test animal.
2. quantity refers to the amount (cc.) of serum injected intravenously into mice.
3. sampling time refers to the time the blood sample was taken from the calf in relation to the injection of the toxin.

The above results are plotted on Charts number IV, V. The charts show that the toxin was removed or disappeared from the blood stream in all cases by 25 minutes post injection. Considerable uniformity can be seen in the length of time required for the toxin to disappear from the blood stream. In five out of the six calves, the sera, at the .2 cc. level of injections, killed mice twice as long as at the .1 cc. level.

Effect on blood counts:

Blood samples were drawn and slides made to study the effect of the toxin on the blood constitutions.

A direct correlation between the values can be seen. There is an increase in total leukocyte counts in all six animals at the thirty minute and three hour mark and a decrease towards normal at the end of twelve hours. The polymorphonuclear cells and lymphocytes responded in a consistent manner with the percentage of polymorphonuclear cells (or neutrophils) decreasing in thirty minutes, increasing in three hours, and decreasing again in twelve hours. Conversely, the percentage of lymphocytes increased in thirty minutes, decreased in three hours, and increased again in twelve hours. The monocytes reacted in an erratic manner, likely due to errors in blood counting. (See Charts VI and VII.)

CHART IV. Persistence of Intravenously Injected Toxin in Calves' Sera as Tested by the Death of Mice

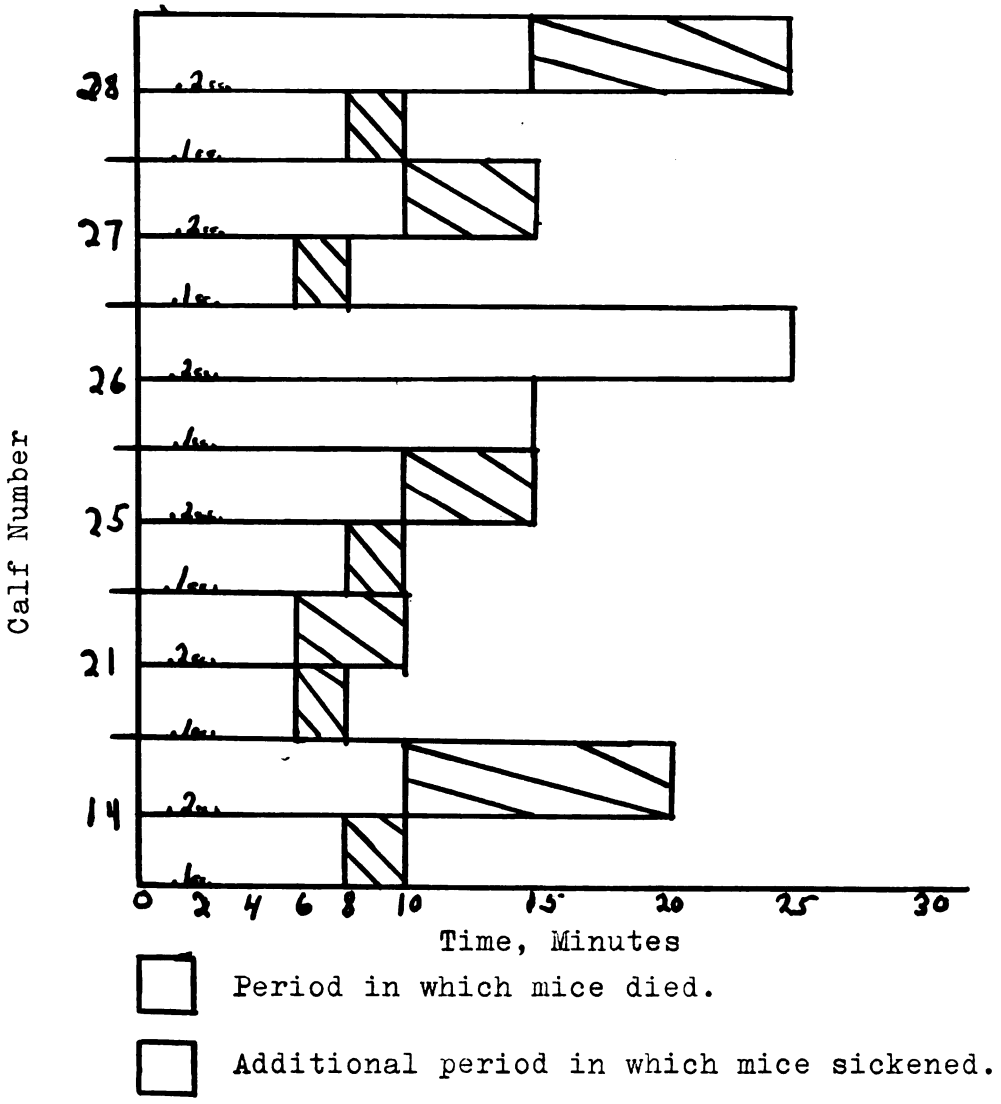
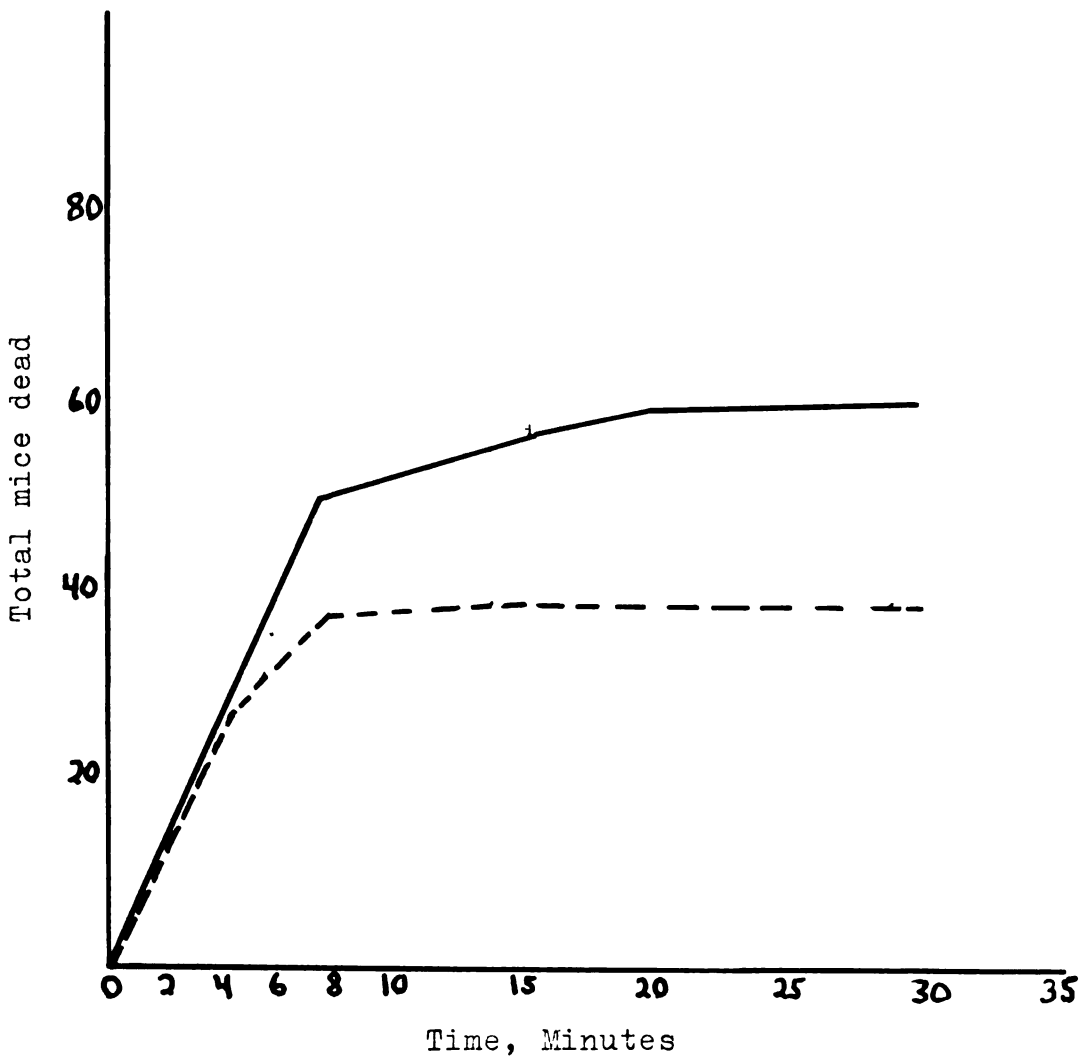


CHART V. Total Mice Dead and Rate of Dying



.1 cc. level - - -

.2 cc. level ———

TABLE V. Effect of Toxin on Differential Blood Count in Calves

Calf Number		Sample Time			
		Prior	30 min.	3 hour	12 hour
14	W.B.C. mm^3	6,950	7,650	8,650	6,800
	Poly. %	8	6	40	10
	Lym. %	86	88	56	84
	Mono. %	6	6	4	6
21	W.B.C. mm^3	9,400	11,050	14,350	11,450
	Poly. %	24	15	36	15
	Lym. %	70	83	59	81
	Mono. %	6	2	5	4
25	W.B.C. mm^3	8,750	8,800	13,650	11,750
	Poly. %	22	17	32	28
	Lym. %	65	77	62	69
	Mono. %	13	6	6	3
26	W.B.C. mm^3	8,750	10,800	13,750	13,450
	Poly. %	48	28	50	50
	Lym. %	46	64	47	45
	Mono. %	5	7	4	5
	Eos. %	1	1		
27	W.B.C. mm^3	14,450	17,450	21,950	14,000
	Poly. %	61	45	78	75
	Lym. %	33	41	17	20
	Mono. %	6	14	5	5
28	W.B.C. mm^3	11,000	18,850	18,650	20,150
	Poly. %	22	23	64	44
	Lym. %	68	73	22	52
	Mono. %	8	3	4	4
	Eos. %	2	1		

CHART VI. Leukocyte Counts Following Toxin Administration

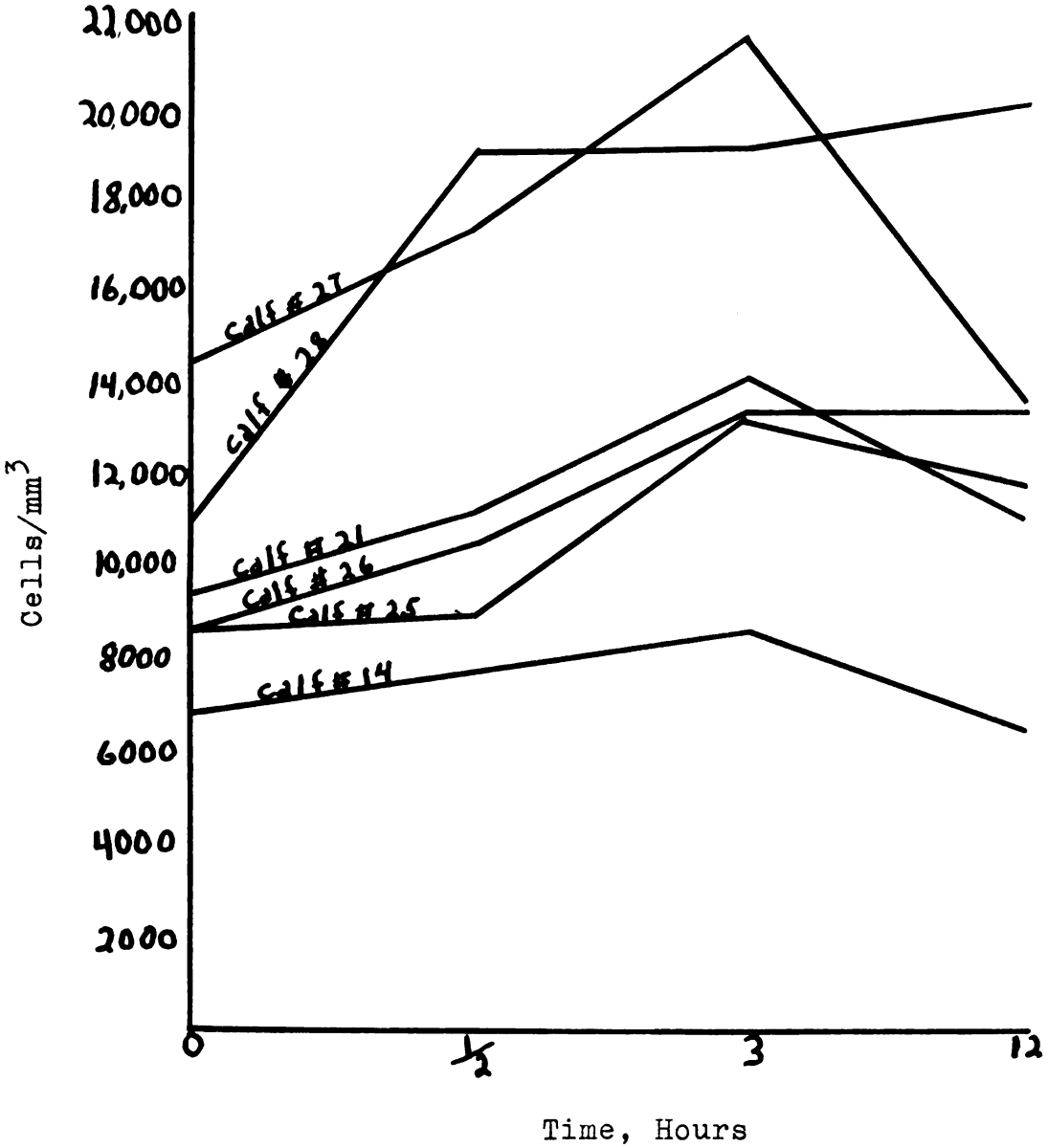
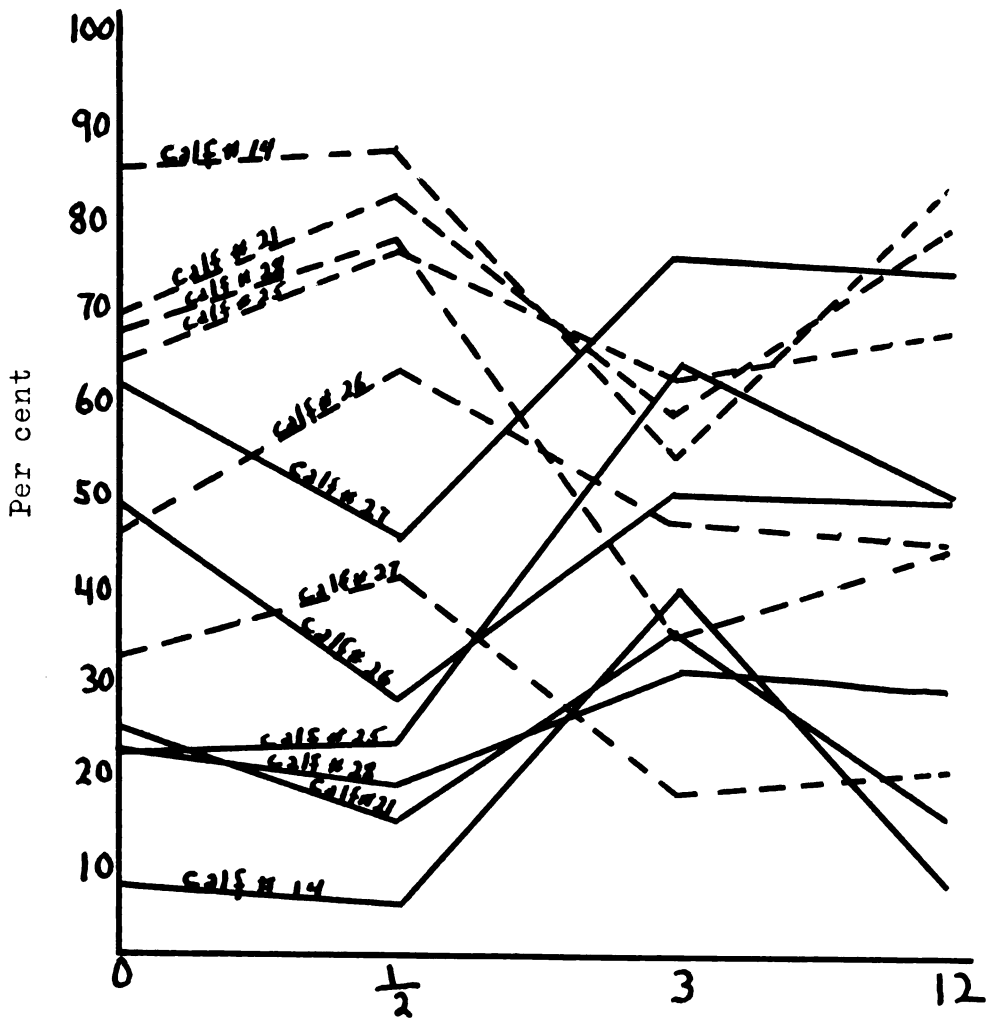


CHART VII. Variation of Polymorphonuclear Cells and Lymphocytes as a Result of Toxin Administration



Decreasing hematocrit readings were noted on samples drawn at thirty minutes and three hours post injection on all of the calves. Likewise, the samples drawn at twelve hours showed an increase in all cases.

TABLE VI. Effect of Toxin on Packed Cell Volume in Calves

	Sample Time			
Calf Number	Prior	30 min.	3 hour	12 hour
14	51%	50%	42%	48%
21	37%	36%	35%	35%
25	44%	34%	33%	38%
26	29%	28%	28%	32%
27	44%	43%	42%	45%
28	55%	54%	48%	50%

Plotting the above results on a graph gives the following picture: (See Chart VIII.)

Urine tests:

Urine samples were collected prior to injection of toxin and during the experiment as it was voided. Samples were kept separate, and urine was injected intravenously into mice at 0.1 cc. and 0.2 cc. level to check for a lethal factor.

CHART VIII. Packed Cell Volume Variation due to Toxin Administration

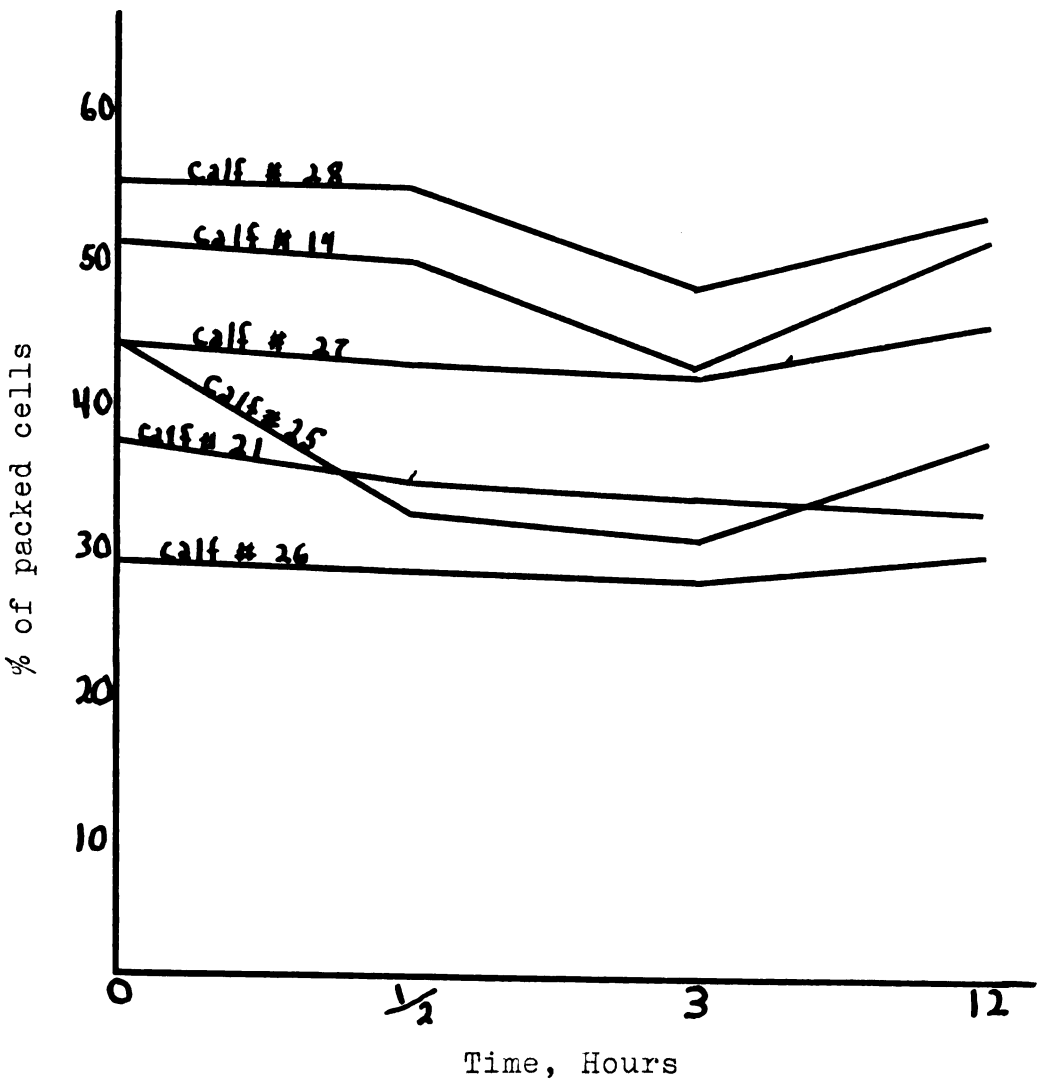


TABLE VII. Urine Study[1].

Sample time[2]	Calf Number											
	14		21[3]		25		26		27		28	
	.1	.2	.1	.2	.1	.2	.1	.2	.1	.2	.1	.2
Prior	00	00	xxx	xxx	00	00	00	00	00	00	00	00
5 min.	00	00	xxx	xxx					00	00		
10 min.	00	00			00	00	00	00			00	00
30 min.	00	00			00	00						
1 hour											00	00
2 hour	xx	xx					00	00	xx	xx	xxx	xxx
2 1/2 hour							xx	xx	00	00		
3 hour	00	00			xx	xx	00	00	00	00	000	000
4 hour	00	00			00	00	00	00	00	00		
Lethal urine + antiserum[4]	~~00~~	~~00~~			0~~0~~	~~0~~x	00	0~~0~~	0~~0~~	0x	00	~~0~~x
Lethal urine + saline[5]	xx	xx		xx	xx	xx	xx	xx	xx	xx	xx	xx
Antitoxin[6]							000~~0~~		00xx			

0 = live mice
~~0~~ = sick mice
x = dead mice

1. Urine was injected intravenously into mice at .1 and .2 cc. quantities in search of a lethal factor.
2. Samples were collected as they were voided and the times are somewhat approximated.
3. Not able to use, since a lethal factor was already present in the urine.
4. Lethal urine mixed with Clostridium perfringens antisera in a 1:1 dilution.
5. Lethal urine diluted with saline in a 1:1 ratio.
6. Clostridium perfringens antitoxin was injected intraperitoneal twenty-four hours prior to the injection of the lethal urine intravenously.

The appearance of a lethal factor in the urine near the two to three hour collection is quite significant as a possible route of toxin excretion. This lethal factor could be neutralized or reduced in potency by combining the urine with Clostridium perfringens antitoxin. No reduction in potency was noted when the urine was diluted with saline. It should be noted that antiserum protected the mice to some degree in the one case in which it was tried. Therefore, it appears that the toxin is excreted by the kidneys in a relatively unchanged form, two to three hours post injection.

PART V

SUMMARY AND CONCLUSIONS

In preliminary work on this problem, it was determined that Clostridium perfringens type C toxin would kill goats and mice but not calves. The routes of administration tried in calves were intravenous, oral, and direct injection into the intestinal tract. However, if the toxin was combined with trypsin, death could be produced.

The symptoms shown upon injection of toxin into calves were as follows: coughing, lacrimation, colic, urination, slight respiratory distress, temperature increase of one degree, heart and respiratory rate doubled, decrease in packed cell volume, increase in the total leukocyte count, and an inverse relationship between the percentage of neutrophils and lymphocytes.

Blood samples drawn from the jugular vein of calves after injection of 15,000 mouse MLD/100 pounds, showed the toxin to be removed from the blood stream within twenty-five minutes of its injection.

A substance lethal to mice appearing in urine samples collected two to three hours after toxin injection, suggests that the toxin is excreted in a relatively unchanged form.

BIBLIOGRAPHY

1. Baldwin, E.: Clostridial Enterotoxemia. Vet. Med., 54, (1959): 123.

2. Barner, R. D.: Hemorrhagic Enterotoxemia. Dept. of Surg. and Med., College of Vet. Med., M.S.U., E. Lansing, Mich. Class Handout. 1961.

3. Beck, C. C., Ellis, D. J.: Enterotoxemia of Bovines. Department of Surgery and Medicine, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan. Unpublished data. 1958.

4. Binkley, F. W.: Personal Communication. Research Clinician, Haver-Lockhart Lab., Kansas City, Missouri.

5. Blood, D. C., Helwig, D. M.: Enterotoxemia of Calves. Aust. Vet. J., 33, (1957): 144.

6. Bosworth, T. J.: A new Type of Toxin Produced by Clostridium welchii. J. Comp. Path., 53, (1943): 245-255.

7. Breed, R. S., Murray, E. G. D., Hitchens, A. P.: Bergey's Manual of Determinative Bacteriology. 7th ed. The Williams and Wilkins Co., Baltimore, Maryland. 1957.

8. Bullen, J. J., Batty: Experimental Enterotoxemia of Sheep. The Effect on the Permeability of the Intestine and the Stimulation of Antitoxin Production in Immune Animals. J. Path. and Bact., 73 (1957): 511-518.

9. Frank, F. W.: Clostridium perfringens Type B from Enterotoxemia in Young Ruminants. Am. J. of Vet. Res., 17, (1956): 492-494.

10. Gregory, D. W.: Clostridium perfringens Type C. Enterotoxemia in a Calf. North Amer. Vet., 37, (1956): 195.

11. Griner, L. C.: "C" Toxoid Available. Rocky Mountain Vet., 1, (1953): 13.

12. Griner, L. A.: Enterotoxemia--A Review of Types of Autointoxication in Domestic Animals. Am. J. of Vet. Science, Feb. 1, 1958: 27.

13. Griner, L. A.: Enterotoxemia of Sheep. I. Effect of Clostridium perfringens Type D Toxin on the Brains of Sheep and Mice. Am. J. of Vet. Res., 22, (1961): 429-441.

14. Griner, L. A.: Enterotoxemia of Sheep. III. Clostridium perfringens Type D Antitoxin Titers of Normal, Nonvaccinated Lambs. Am. J. of Vet. Res., 22, (1961): 447-448.

15. Griner, L. A.: Hemorrhagic Enteritis. Diseases of Cattle. American Vet. Publication, Evanston, Ill., (1956): 573.

16. Griner, L. A., Archelman, W. W., Brown, G. D.: Clostridium perfringens Type D (Epsilon) Enterotoxemia in Brown Swiss Dairy Calves. J.A.V.M.A., 129, (1956): 375-376.

17. Griner, L. A., Baldwin, E.: Further Work on Hemorrhagic Enterotoxemia of Infant Calves and Lambs. Proc. Book A.V.M.A. 91 meeting, (1954): 45.

18. Griner, L. A., Bracken, F. K.: Clostridium perfringens (type C) in Acute Hemorrhagic Enteritis of Calves. J.A.V.M.A., 122, (1953): 99.

19. Griner, L. A., Carlson, W. D.: Enterotoxemia of Sheep. II. Distribution of I^{131} Radioiodinated Serum Albumin in Brains of Clostridium perfringens Type D Intoxicated Lambs. Am. J. of Vet. Res., 22, (1961): 443-446.

20. Griner, L. A., Johnson, H. W.: Clostridium perfringens Type C in Hemorrhagic Enterotoxemia of Lambs. J.A.V.M.A., 125, (1954): 125-127.

21. Hagan and Bruner: The Infectious Diseases of Domestic Animals. 4th ed. Comstock Publishing Associates, Ithaca, New York, 1957.

22. H. C. Burns Co.: Symposium--5th Annual. The Development and Economics of a Sheep Practice, Jan. 31, 1960.

23. Hepple, J. R.: Necrotic Enterotoxemia in a Calf Due to Clostridium welchii Type B. Vet. Record, 57, (1945): 47-48.

24. Keast, J. C., McBarron, E. J.: A Case of Bovine Enterotoxemia. Aust. Vet. J., 30, (1954): 305.

25. Macrae, D. R., Murray, E. G., Grant, J. G.: Enterotoxemia in Young Suckled Calves. Vet. Record, 55, (1943): 203.

26. Merchant, I. A.: An Outline of the Infectious Diseases of Domestic Animals. Burgess Publishing Co., Minneapolis 15, Minn., (1957): 117.

27. Montgomerie, R. F., Rowlands, W. T.: Lamb Dystntery. Vet. J., 90, (1934): 323.

28. Oakley, C. L.: The Toxins of Clostridium welchii. Bull. Hyg., 18, (1943): 781.

29. Oakley, C. L., Ross, H. E., Right, G., Payling, E., MacFarland, M. G., Gorden, W. S., Van Heyninger: Discussion on Clostridial Toxins in Relation to Type Specificity for Different Species of Host. Pro. Roy. Soc. Med., 43, (1953): 883-892.

30. Oakley, C. L., Warrack, G.: Routine Typing of Clostridium welchii. J. Hyg., 51, (1953): 102-107.

31. Rose, A. L., Edgar, G.: Enterotoxemia Jaundice of Sheep and Cattle. Australian Vet. J., 12, (1936).

32. Schofield, F. W.: Enterotoxemia (Sudden Death) in Calves Due to Clostridium welchii. J.A.V.M.A., 126, (1955): 192.

33. Shanks, P. L.: Suspected Enterotoxemia in Cattle. Vet. Record, 57, (1945): 47-48.

34. Shirley, G. N.: Clostridium Enteritis in Cattle. Vet. Record, 70, (1958): 478-480.

35. Smith, H. A., Jones, T. C.: Enterotoxic Hemorrhagic Enteritis. Veterinary Pathology, Lea & Febiger, Philadelphia, Pa., (1957): 754.

36. Sollmann: A Manual of Pharmacology. 8th ed. W. B. Saunders Co., Philadelphia, Pa., (1957).

37. Stableforth and Galloway: Diseases Due to Bacteria. I, (1959). Academic Press, Inc. Publishers, New York, N.Y.

38. Van Heyninger, W. E.: Bacterial Toxins. Blackwell Publications, Oxford, England, (1950).

39. Corn States Advertisement for Toxoid and Antitoxin. Corn States Laboratories, Inc., Omaha 8, Nebraska.

40. Hemorrhagic Enterotoxemia of Calves. Corn States Needle, Fall (1954): 3-5.

41. Sera for the Identification of Clostridia. Wellcome Research Lab., Beckenham, England.

42. Seven Products to Combat Clostridium perfringens. Haver-Lockhart Lab. "Messenger," Sept.-Oct., (1957).

43. Use of Antibiotics Against Clostridium perfringens. Annual Review of Micro., 7, (1953): 236.