MONTANA STATE UNIVERSITY

The hemolysins of Clostridium hemolyticum

by Edgardo A Lozano

A thesis submitted to the Graduate Faculty in partial fulfillment of "the requirements for the degree of DOCTOR OF PHILOSOPHY in Bacteriology
Montana State University
© Copyright by Edgardo A Lozano (1965)

Abstract:
Studies with toxic culture fluids of Clostridium hemolyticum showed that the fluids reacted with egg yolk) lecithin and sphingomyelin. The reactions with these substrates were manifested by an initial opalescense -which eventually resulted in the production of a curd of fat. Phosphoryl-choline was released from the' reaction mixtures indicating that the organism produced lecithinase C. Mixing the toxic fluids with erythrocytes of various species resulted in hemolysis of the cells. The lytic patterns with mouse erythrocytes were essentially linear while those with chicken erythrocytes were irregular and characterized by two slopes of hemolysis with a flat plateau between these reactions. The erythrocytes of mice ,, were much more susceptible to hemolysis than those of chickens, but the two species were about equally susceptible to the effects of the toxic fluids. All the reactions could be effectively neutralized by C hemolyticum and Clostridium novyi type B antitoxins.

From the proceeding studies it was assumed that the culture fluids contained more than one hemolytic factor, and because of this, the crude culture fluids were fractioned electrophoretically. The toxic culture fluids could be fragmented into seven separate entities which were not present in the uninoculated culture medium. The four slower moving constituents were hemolytic and were identified as a proteinase, two phospholipases and a lipase. These substances were not equally hemolytic to erythrocytes of different species, and with the exception of the lipase were serologically similar. The proteinase was the slowest moving entity, hydrolyzed emulsions of X-ray film, and was not obtainable in pure form.. The phospholipases moved faster and hydrolyzed both lecithin and sphingomyelin. One of these two substance was necrotizing and was produced in greater amounts than the other hemolysins. The lipase hydrolyzed sorbitan trioleate and was the slowest moving hemolysin.

THE HEMOLYSINS OF CLOSTRIDIUM HEMOLYTICUM

by

EDGARDO A. LOZANO

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Bacteriology

Approved:

Head, Major Department

Chairman, Examining Committee

Dean, Graduate Division

MONTANA STATE COLLEGE
Bozeman, Montana

June, 1965

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to the members of the Botany and Bacteriology Department of Montana State College.

Special acknowledgement is made to Dr. Louis DS. Smith for advice and constructive criticism; to Dr. Gary A. Strobel for guidance in the electrophoresis work; and to my wife Elvia, for encouragement.

The author also expresses his appreciation to the National Institutes of Health for the fellowship granted under their Training Program in Microbiology.

TABLE OF CONTENTS

	Page
List of tables	vii
List of figures	ix
Abstract	x
INTRODUCTION AND REVIEW OF THE LITERATURE	1
History of agent responsible for bacillary hemoglobinuria	1
Toxic and serologic properties	2
Phospholipases of C. hemolyticum and other bacteria	3
Mechanisms of toxicity and enzyme activity	5
Experimental basis for the thesis	6
MATERIALS AND METHODS	10
Selection of organism for toxin production	10
Characteristics of the strain	10
Culture media used	12
Stock culture media	12
Toxin production media	14
Method for preparation and storage of toxic materials	17
Method for estimating optical density of proteins in acrylamide gels	18
Method for the preparation and storage of egg yolk suspension	19
Method for the preparation of lecithin	19
Method for obtaining and preparing erythrocyte suspensions	20
Method for the preparation of diluents for toxic materials	21
Method for estimating lecitho-vitellin (LV) reaction	22

Page

Method for estimating *in vitro* hemolysis 23

Method for determining liberated phosphorylcholine 24

Methods for estimating toxicity and gross pathology 25

Method for determining proteinase activity 27

Methods of estimating lipase activity 29

Serological procedures 30

Development of standards 30

Serological tests with purified toxins 30

Method of conducting disc gel electrophoresis 31

Staining gel column and developing "cutting patterns" .. 31

Collecting and preserving purified toxins 33

EXPERIMENTAL RESULTS - SECTION I 34

I. Work with crude toxin solutions 34

Identification and characteristics of *C. hemolyticum* 7170 34

Morphological and cultural characteristics 34

Studies on toxic culture fluids 35

Toxin production in four media 35

Verification of purity 39

Effect of pH on LV reaction 39

Effect of calcium ions on the LV reaction 40

Liberation of acid soluble phosphate from egg yolk . 42

Hemolysis pattern with chicken and mouse erythrocytes 42

Page

Lethality assays 44

Gross pathology of inoculated animals 49

Effects of aging and concentration procedures 50

Brief summary of results with toxic culture fluids 50

EXPERIMENTAL RESULTS - SECTION II 54

II. Experiments with partially purified toxins 54

Fractioning of toxic culture fluids 54

Biochemical characteristics of isolated hemolysins 58

Heat inactivation studies 58

Serologic studies of purified toxin 58

Hydrolysis of specific substances 61

Liberation of phosphorycholine from phospholipids . 63

Effect of cysteine 66

Effect of EDTA 66

pH optima 68

Hemolytic range of isolated enzymes 71

Identification of a proteinase 71

Some studies on the isolated proteinase 74

Identification of a lipase 74

Necrotizing and lethality potential of the hemotoxins 78

DISCUSSION 80

SUMMARY 86

LITERATURE CITED 89

LIST OF TABLES

Table		Page
1.	Some characteristics of C. hemolyticum 7170 (Physiology)	36
2.	Some characteristics of C. hemolyticum 7170 (Toxin neutralization)	37
3.	C. hemolyticum toxin tests	38
4.	Effect of pH on LV reaction (Batch 4B)	40
5.	Effect of calcium ion on LV reaction	41
6.	Liberation of phosphate from egg yolk	43
7.	Hemolysis and liberation of phosphate from erythrocytes by C. hemolyticum toxic fluid	47
8.	Titrations of two culture fluids in chickens and mice	48
9.	Effect of culture aging on toxin harvest	51
10.	Effect of concentration procedure on toxin activity	52
11.	Heat inactivation studies of partially purified toxins of C. hemolyticum	60
12.	Summary of serological studies	62
13.	Liberation of acid soluble phosphate from egg yolk with partially purified toxins of C. hemolyticum	64
14.	Effect of partially purified toxins on lecithin and sphingomyelin	65
15.	Cysteine inactivation studies	67
16.	Calcium requirement of C. hemolyticum toxins	69
17.	pH studies of partially purified toxins of C. hemolyticum	70
18.	Hemolysis range of isolated toxins	72

Table		Page
19.	Hemolysis range of isolated toxins; "Hot-cold" effects	73
20.	Experiments with proteinase (Band #2); effects of EDTA and trypsin inhibitor on X-ray emulsion	76
21.	Lipase activity of partially purified toxins of C. hemolyticum	77
22.	Effect of intracutaneous and intravenous inoculations of isolated toxins	79

LIST OF FIGURES

Figure		Page
1.	Cellophane sac culture system	16
2.	Patterns of hemolysis on chicken erythrocytes with five different C. hemolyticum toxic fluids	45
3.	Patterns of hemolysis on mouse erythrocytes with five different C. hemolyticum toxic fluids	46
4.	Drawings of acrylamide gel columns of toxic fluid 7170-V and medium control	57
5.	Relative optical density of C. hemolyticum proteins in an acrylamide gel column	59
6.	Proteinase test of C. hemolyticum 7170 band 2	75

ABSTRACT

Studies with toxic culture fluids of Clostridium hemolyticum showed that the fluids reacted with egg yolk, lecithin and sphingomyelin. The reactions with these substrates were manifested by an initial opalescense which eventually resulted in the production of a curd of fat. Phosphorylcholine was released from the reaction mixtures indicating that the organism produced lecithinase C. Mixing the toxic fluids with erythrocytes of various species resulted in hemolysis of the cells. The lytic patterns with mouse erythrocytes were essentially linear while those with chicken erythrocytes were irregular and characterized by two slopes of hemolysis with a flat plateau between these reactions. The erythrocytes of mice were much more susceptible to hemolysis than those of chickens, but the two species were about equally susceptible to the effects of the toxic fluids. All the reactions could be effectively neutralized by C. hemolyticum and Clostridium novyi type B antitoxins.

From the preceeding studies it was assumed that the culture fluids contained more than one hemolytic factor, and because of this, the crude culture fluids were fractioned electrophoretically. The toxic culture fluids could be fragmented into seven separate entities which were not present in the uninoculated culture medium. The four slower moving constituents were hemolytic and were identified as a proteinase, two phospholipases and a lipase. These substances were not equally hemolytic to erythrocytes of different species, and with the exception of the lipase were serologically similar. The proteinase was the slowest moving entity, hydrolyzed emulsions of X-ray film, and was not obtainable in pure form. The phospholipases moved faster and hydrolyzed both lecithin and sphingomyelin. One of these two substances was necrotizing and was produced in greater amounts than the other hemolysins. The lipase hydrolyzed sorbitan trioleate and was the slowest moving hemolysin.

INTRODUCTION AND REVIEW OF THE LITERATURE

History of agent responsible for bacillary hemoglobinuria.

Clostridium hemolyticum is best known as the micro-organism responsible for bacillary hemoglobinuria of cattle. The disease was probably known as early as 1885 (Vawter and Records, 1926; Records and Vawter, 1945) but no organized efforts to control it were undertaken until it had reached epizootic proportions in some mountain valleys of Nevada (Records and Vawter, 1945). The Agricultural Experiment Station at the University of Nevada initiated studies on the disease in 1914 (Records and Vawter, 1945). Failure to isolate the etiological agent limited the study to geographical distribution, clinical symptoms and gross pathology of the malady. The disease was described by Meyer (1916), Mack and Records (1917) and Records and Vawter (1921).

C. hemolyticum was finally isolated and identified in 1926 by Vawter and Records. The large Gram-positive rods form oval spores which swell the sporangium and are usually located subterminally The Gram-positive reaction is generally seen in young cultures only, and cultures older than 24 hours contain mostly Gram-negative organisms. The rods have rounded ends, measure approximately 1.0 u by 3-- 5 u, and are sluggishly motile by means of peritrichous flagella.

Colonies growing on blood agar plates are small, grayish and surrounded by zones of partial hemolysis. The size of the colonies and their zones of hemolysis are dependent on a number of factors such as length of incubation and type of medium. Subsurface inoculation into glucose-free media results in colonies that are initially lenticular but which

become wooly and develop short filamentous edges on prolonged incubation. Some strains form "snowflake" type colonies which are considered to be rough types. These bacteria are fully virulent but unsuitable for agglutination because they are unstable when used as antigens.

C. hemolyticum ferments glucose and fructose but not galactose, maltose, lactose, sucrose, mannitol, dulcitol, arabinose, xylose, dextrin, insulin and salicin. There are variants of the organism that ferment glycerol (Smith, 1953) and maltose (Smith, Claus and Matsuoka, 1956). The organism liquefies gelatin in two to six days, and acidifies and coagulates milk slowly. Hydrogen sulfide and indole are formed, nitrates are not reduced, and the methyl red and Vogues-Proskauer tests are negative. Coagulated serum and egg albumin are not digested.

Toxic and serologic properties.

At the time C. hemolyticum was isolated it was found that the organism produced a potent hemolytic toxin which was responsible for the massive destruction of erythrocytes in affected animals (Vawter and Records, 1926). These workers showed that the organism produced a separate necrotizing substance which persisted long after the hemolysin had disappeared from culture fluids that were allowed to remain in the incubator. The necrotizing substance described by Vawter and Records was not detected by Jasmin (1947). He found that C. hemolyticum produced a single substance that was both necrotic and hemolytic. Jasmin also observed that the toxin caused opalescence and liberation of fat from egg yolk suspensions. This reaction was similar to the lecithovitellin reaction (LV) of

C. perfringens type A. Because of these similarities he suggested that the C. hemolyticum toxin was a lecithinase C. His suggestion was supported by Macfarlane (1950a) who demonstrated that the toxin hydrolyzed lecithin and sphingomyelin but not cephalin type compounds. The products of the hydrolysis were free fats and phosphorylcholine.

Vawter and Records (1929) demonstrated that the lethal effects of the toxin produced by these organisms could be inhibited by treatment with specific antiserum. Jasmin (1947) showed that the in vivo and in vitro effects of the toxin could be neutralized with sera prepared against C. hemolyticum but not with sera prepared against C. perfringens type A. He also observed that the alpha toxin of C. perfringens could not be neutralized by C. hemolyticum antitoxin. The serological disimilarity between these lecithinases was verified by Oakley, Warrack and Clarke (1947), who also found that C. hemolyticum toxin was serologically related to the beta toxin of C. novyi. Because of the similarity, Oakley and Warrack (1959) suggested that C. hemolyticum should be considered as type D of C. novyi. The observation that C. hemolyticum also produced a substance similar to the zeta toxin of C. novyi type B supports their suggestion.

C. hemolyticum specifically agglutinates with homologous sera but not with sera prepared against C. perfringens, C. chauvoei, C. septicum or C. novyi (Vawter and Records, 1931).

Phospholipases of C. hemolyticum and other bacteria.

A number of bacteria are known to produce phospholipases of the same

biochemical type as those of C. perfringens and C. hemolyticum. Included in the group are both animal and insect pathogens. Clostridium novyi, as a species, produces more than one phospholipase of the same biochemical type but these substances are serologically different (M. G. Macfarlane, 1942; Oakley, Warrack and Clarke, 1947; M. G. Macfarlane, 1948). Oakley et al. (1947) showed that the lethality of culture filtrates of C. novyi is caused mainly by their content of alpha toxin rather than by their lecithinases C which are usually produced in small amounts. Clostridium bifermentans also produces a toxin that reacts with egg yolk (Hayward, 1943) and the substance was identified as a lecithinase C (Miles and Miles, 1947; 1950). These workers demonstrated that enzymically equivalent concentrations of the substance are much less lethal than the alpha toxin of C. perfringens.

Bacillus cereus produces a substance that reacts with egg yolk (McGaughey and Chu, 1948). The substance was identified as a lecithinase C (Chu, 1949) that is pathogenic to the larvae of the larch saw fly (Heimpel, 1955; Kushner and Heimpel, 1957). Bacillus anthracis also produces a substance that reacts with egg yolk (Ruata and Caneva, 1901; McGaughey and Chu, 1948) and with human serum (Nagler, 1939). This substance was identified as a lecithinase C (Costlow, 1958).

There are some bacteria which produce substances, other than lecithinase C, that react with egg yolk. McClung and Toabe (1947) mentioned that egg yolk reactions are seen with some of the species of Actinomyces, Aspergillus and Bacillus. Clostridium sporogenes reacts with egg yolk, but the reaction is not typical of lecithinase C (McClung and

Toabe, 1947). Egg yolk reactions were also seen with Listeria monocytogenes (Fuzi and Pillis, 1962). It is not known what substances are produced by these bacteria that cause these LV type reactions.

Coagulase positive strains of Staphylococcus aureus react with egg yolk (Gillespie and Adler, 1952) but the reaction is caused by a lipase and not by a lecithinase C (Shah and Wilson, 1962). Rogols, Fizette and Bohl (1959) reported that the hemolysin and toxin of Leptospira pomona were partially neutralized by lecithin, leading them to believe that the organism produced a lecithinase. It has been shown, however, that the toxin may be a lipase that can hydrolyse wynthetic lipids (Patel et al., 1963).

Mechanisms of toxicity and enzyme activity.

Lecithinases C hydrolyze lecithins and related phospholipids (Macfarlane and Knight, 1941). Because of this hydrolytic activity exposure of erythrocytes to these substances will result in alterations of the stroma and release of hemoglobin. In natural infections of cattle with C. hemolyticum, the disease is characterized by massive intravascular hemolysis of erythrocytes. It has been observed that the erythrocyte count drops from a normal of approximately 6,000,000 per mm^3 to about 1,500,000 per mm^3 shortly before death (Records and Vawter, 1921; Vawter and Records, 1926). The massive destruction of erythrocytes leads to anoxia, damage to endothelial tissues and increased capillary permeability. Because of the increased permeability there is a resultant extravasation of hemoglobin-stained plasma into the abdominal cavity (Records and

Vawter, 1945).

The toxin produced by C. hemolyticum is a lecithinase C which has not been studied as extensively as lecithinase C of C. perfringens. It is not known how C. hemolyticum toxin affects cells other than erythrocytes, but if this lecithinase C is comparable in its toxigenic mechanisms to the alpha toxin of C. perfringens, then it can be inferred that the toxic lecithinase of C. hemolyticum can attack the mitochondria of cells (Macfarlane and Datta, 1954), may interfere with the oxidation of succinate (Wooldridge and Higginbottom, 1938) and may disarrange the magnesium activated adenosinetriphospatase of muscle (Kielley and Meyerhof, 1950).

The mechanism of enzymic action on egg yolk may involve the action of a single enzyme upon a single substrate. This would be true if the culture fluid of C. hemolyticum contained only lecithinase C. If the culture fluids contain lipases or proteinases the yolk reactions could also be caused by these enzymes. It is known that bacterial lipases and proteinases react with yolk suspensions (Shah and Wilson, 1962; Willis and Turner, 1962; Crook, 1942; Kushner, 1957, Willis and Gowland, 1962).

Hemolysis, like the LV reaction, may also be caused by a number of enzymes. However, the main determinant of this reaction appears to be the lecithinase C which is produces in larger amounts than the other enzymes.

Experimental basis for the thesis.

The hemolytic, toxic and lecitho-vitellin (LV) reactive capacity of lecithinases C have been found to be disproportional. While R. G. Macfarlane and associates (1941) and Jasmin (1947) found a direct relationship

between LV, hemolysis and toxicity for filtrates of C. perfringens and C. hemolyticum, Dolby and M. G. Macfarlane (1956) found that different strains of C. perfringens yileded filtrates with varying LV/toxicity ratios and divergent affinities towards erythrocytes of the same species. These workers also indicated that there were no synergistic effects in the reactions and that hemolysis estimates were unreliable unless liberated phosphates were determined concomitantly. In earlier work, M. G. Macfarlane (1950b) found that the serologically different lecithinases C of C. perfringens and C. novyi also differed in their affinites toward erythrocytes of different species. She showed that it required less alpha toxin of C. perfringens to hemolyze sheep erythrocytes than it did to hemolyze horse erythrocytes while the opposite was true for the beta toxin of C. novyi.

Work with other toxic filtrates has also revealed interesting inconsistencies. Miles and Miles (1947) found that filtrates of C. bifermentans lysed mouse and rabbit erythrocytes but not those of horse, human, sheep or guinea pigs. Chu (1959) found that filtrates of B. cereus remained hemolytic when treated with normal horse serum, but the same filtrates lost their LV reactivity. Smith and Gardner (1950) demonstrated disproportionate inactivation of C. perfringens type A filtrates which were exposed to reducing substances; the LV reaction was inactivated faster than the hemolytic reaction. Kushner (1957) found that crude lecithinase C preparations of Bacillus cereus exhibited anomalous heat inactivation which was not seen with more purified enzyme preparations. He also observed that LV type reactions and lecithinase activity differed in their heat inactivation rates, and that the cloudiness of the egg yolk reaction can be caused by a lipase.

The list of substrates hydrolyzable by the lecithinases C has increased over the years. M. G. Macfarlane and Knight (1941) initially found that the lecithinase of C. perfringens hydrolyzed lecithin and sphingomyelin but not phosphatidylethanolamine or phosphatidylserine. Matsumoto (1961) observed hydrolysis of sheep erythrocyte's phosphatidylethanolamine, and de Haas et al. (1961) increased the list to include synthetic cephalin if a small amount of natural lecithin is added to the reactants. As more was learned about the phospholipases, it became apparent that many of the discrepancies could probably be explained by the fact that most of the studies were conducted with impure enzyme preparations. The reactions were usually determined using complex substrates.

Seldom were natural substances for in vitro tests derived from the same species used for toxicity assays. With this thought in mind, the LV reaction using chicken egg yolk for the substrate may be suitable for toxicity estimates of chickens, but any relationship between LV with lethality estimates in other animals may be coincidental. Also hemolytic estimates would probably correlate better with lethality estimates if the erythrocytes used in the tests were obtained from the same species used for lethality titrations.

Preliminary work to determine if there was a correlation between hemolysis and toxicity of culture fluids of C. perfringens type A, Bacillus cereus and C. hemolyticum revealed that there was no relationship between the in vivo and in vitro tests. New-born chickens and adult mice were used for the in vivo titrations, and erythrocytes from the same species were used for the in vitro tests. The unexpected results left two possibilities to

consider. First, studies could be directed towards finding more than one hemolytic substance in each of the preparations and second, studies could be directed to find marked differences in the erythrocyte susceptibility.

It was decided to limit further investigations to work with culture fluid from a single species. Culture fluids of C. hemolyticum were selected because the organism purportedly produces but one lethal hemolytic substance (Jasmin, 1947). No interference was expected with a lipase that this organism produces (Oakley and Warrack, 1959) because the enzyme is non-hemolytic, non-lethal and non-necrotic.

The present thesis is composed of two parts. The first section is concerned with work with crude toxins which were used to explore the relationship between hemolysis, LV and lethality of culture fluids. The second section deals with work with partially purified toxins.

MATERIALS AND METHODS

Selection of organism for toxin production.

A toxigenic strain of Clostridium hemolyticum catalogued by the accession number 7170 of the Veterinary Research Laboratory of Montana State College was used for toxin production. Throughout this thesis the culture will be referred to as "Strain 7170". The initial isolation was made on October 16, 1960 from the liver of a cow which had died of bacillary hemoglobinuria. The infected tissues were collected by Drs. R. D. Read and R. C. Keyser of Ronan, Montana and the isolation and identification was made by a staff bacteriologist of the Veterinary Research Laboratory.

Characteristics of the strain. Strain 7170 was identified by a number of tests, including morphology, toxin production and serum neutralization. The organism is a Gram-positive sporulating rod which swells slightly as it sporulates. Actively growing cultures generally become Gram-negative in less than 24 hours incubation at 37 C. Motility is not marked and is best demonstrated in semisolid agar rather than by the hanging drop technique.

To determine carbohydrate fermentations, various sterilized carbohydrates were added to tubes of freshly prepared thioglycollate medium (BBL 01-136C) to give 0.5% concentration of each of the sugars. After these additions, the tubes were boiled for a few minutes to drive off oxygen and to mix the added carbohydrates with the thioglycollate broth. After cooling, the tubes were inoculated with 0.1 ml of culture using pipettes fitted with small rubber bulbs. The tubes were incubated for four days anaerobically in Brewer jars at 37 C. Strain 7170 actively fermented

glucose but not maltose, lactose, sucrose or salicin.

Inoculated tubes of iron milk showed no apparent reaction after four days of incubation, but a soft clot formed on incubation for seven days. Gelatin was liquified in four days, and media containing meat were blackened if steel wool was added as an aid for anaerobiosis. No tests were conducted for nitrate reduction or formation of indole.

Colonial morphology was determined from growth on freshly poured bovine blood agar plates. During incubation for 72 hours at 37C the organism formed small raised colonies with entire margins. The small glossy colonies were transparent, grayish, had very soft consistency, and were surrounded by zones of partial hemolysis which extended two to three mm beyond the edges of the colonies. No growth was obtained on egg yolk plates prepared with blood agar base, but heavily inoculated areas showed whitish discoloration which was probably caused by lecithinase C carry-over from broth cultures used as inoculum.

Intramuscular inoculation of actively growing cultures killed guinea pigs, mice and one to three-day old baby chickens. Guinea pigs usually died in 48 hours when injected with cultures that had been transferred a number of times on laboratory media, but the reaction could be shortened to about 24 hours with cultures transferred serially in animals. Mice and baby chickens were usually killed in less than 24 hours and both of these species exhibited "bleeding" from body orifices. Stock cultures were prepared from organisms growing in tissues of infected guinea pigs; this increased the toxigenicity of Strain 7170.

Preliminary neutralization tests were carried out with fluids of 18

hour cultures grown in liver infusion medium. The cultures were centrifuged at 12,100 x g for 15 minutes and 1.0 ml of the supernatant fluids was placed in 85 X 15 mm test tubes together with 1.0 ml of a solution of antitoxin* containing 100 units and a drop of 0.2 M calcium chloride. The mixture was shaken vigorously for a few seconds and was then incubated at room temperature for 40 minutes. One ml of a yolk suspension was added to each tube which was again vigorously shaken and incubated at 37 C for 45 minutes. Control tubes for these neutralization tests included samples without antitoxin, and samples without toxin. Neutralization of toxin was manifested by inhibition of opalescence of the egg yolk suspension.

Culture media used.

Stock culture media. Two media were used for the maintenance and preservation of the organism. Ground beef medium was used for routine transfers and storage for periods which did not exceed about a month. This medium had a liquid phase composed of liver infusion to which was added 1% trypticase (BBL 02-148), 0.5% yeast extract (Difco 0 127-01), 0.2% soluble starch (Argo Div. Corn Products Co.) and 0.01% magnesium sulfate. The liver infusion was prepared by mixing 1 lb of ground liver in 1000 ml water. The ground meat and water were placed in a stainless steel container and were boiled for five minutes. The resultant broth and coagulated meat were cooled by placing the metal container in a bath of running cold tap water and then in a refrigerator. After about four hours, congealed fats

*Diagnostic serum produced by the Wellcome Research Laboratories, Beckenham, England.

and meat residue were removed by straining through cheese cloth. The broth was clarified by centrifugation at 2520 x g for 15 minutes. The ingredients listed above were added to the supernatant fluid and the reaction was adjusted to pH 7.6 with 1 N NaOH. This medium was placed in test tubes which contained a small ball of steel wool and about $\frac{1}{2}$ inch of ground cooked lean beef. Before dispensing the medium, a single tube was tested for final pH. If the reaction dropped below pH 7.4, more sodium hydroxide was added to the infusion and a second tube was tested. The procedure was repeated until a pH of 7.4 to 7.6 was achieved. The tubes were then sterilized by autoclaving at 121 C for 20 minutes.

Since the medium was used for periods not to exceed one week after preparation, it was necessary to prepare it in small batches. Alternatively the ground beef used in the medium was cooked and then preserved by freezing at -20 C. The liver infusion was preserved by refrigeration at 6 - 8 C after 1% chloroform had been added to prevent microbial growth. While the meat would last for long periods of time the chloroformed infusion would remain usable for only about two to three months. Ground beef medium was usually prepared in 100 ml amounts which was enough for approximately 10 to 12 tubes.

Brain medium was used for routine maintenance and prolonged storage of Strain 7170. The medium was prepared by adding an equal volume of distilled water to fresh bovine brain and boiling the mixture for 15 minutes. The cooked tissues were then transferred to a Waring blendor for a few seconds to chop the tissue into small particles. Polypeptone (BBL 02-149) was added to make a two percent W/V solution, the reaction adjusted to

pH 7.3 with 5 N NaOH, and the medium dispensed into screw-cap tubes containing small balls of steel wool. The tubes were placed in a bath of boiling water, shaken gently to drive off excess air and then were autoclaved for 30 minutes at 121 C. The tubes were either used immediately upon cooling or were frozen at -20 and subsequently boiled and cooled prior to incubation.

Toxin production media. Three media were used for production of toxin. A "digest medium" was prepared by digesting liver and stomach tissue of hogs and muscle tissue of beef. One thousand grams of washed, ground hog stomach; 500 g of hog liver; 500 g of ground lean beef and 2000 ml of distilled water were placed in a container and thoroughly mixed. The reaction of the mixture was lowered to pH 2.0 with concentrated hydrochloric acid. Ten grams of pepsin were added and mixed into the acidified tissues which were then digested for 16 hours at 55 C. After the first four hours the pH was readjusted to 2.0. The mass of digested tissue was boiled for 30 minutes to inactivate residual pepsin. The digest was then cooled with the aid of running tap water, clarified by centrifugation at 2520 x g for 20 minutes, and the supernatant fluid placed in a glass container for storage at -20 C. Prior to use the digest was diluted 1:1 with distilled water, the pH adjusted to 7.2 and the diluted digest placed in suitable containers for sterilization by autoclaving for 30 minutes at 121 C. Because this medium produced false LV type reactions, it was necessary to cultivate Strain 7170 in saline filled cellophane sacks immersed in the medium, which was dispensed into Mason jars. A number of glass tubing outlets were provided for inoculation of fluids into the sacks and for pressure

compensation during autoclaving. Similar outlets for decompression of the medium were also provided. The top assembly of the jars was covered with cotton and gauze and the finished assemblies were autoclaved for 30 minutes at 121 C. (Fig. 1 represents a drawing of this assembly).

Following autoclaving the medium was colled to room temperature by placing the culture assemblies in baths of running cold tap water. The cooled assemblies were then placed inside Brewer jars which were made anaerobic by repeated careful displacement of gasses within the jars with hydrogen. Care was taken not to exceed a vacuum of 15 inches of Hg in the displacement process which was repeated four times. After allowing a period of 24 hours for diffusion of nutrients into the cellophane sacks, the culture assemblies were taken out of the Brewer jars and the saline sacks inoculated with actively growing cultures of C. hemolyticum. The assemblies were once again replaced inside the anaerobe jars and incubated for 16 hours at 37 C.

An "infusion medium" of pork was composed of 3% trypticase (BBL 02-148), 1% yeast extract, .02% L-cysteine, 0.25% Na_2HPO_4, and 0.125% glucose. The infusion for this medium was prepared with pork liver and muscle meats using 2/3 lb liver and 1/3 lb lean muscle per liter of water. The mixture of meats was ground, water was added and the suspension boiled for five minutes. The resultant broth was cooled and refrigerated to solidify the fats which were removed with a stainless steel screen. The broth was filtered through cheese cloth and clarified by centrifugation at 2520 x g for 15 minutes.

The infusion with the additives listed above was dispensed into

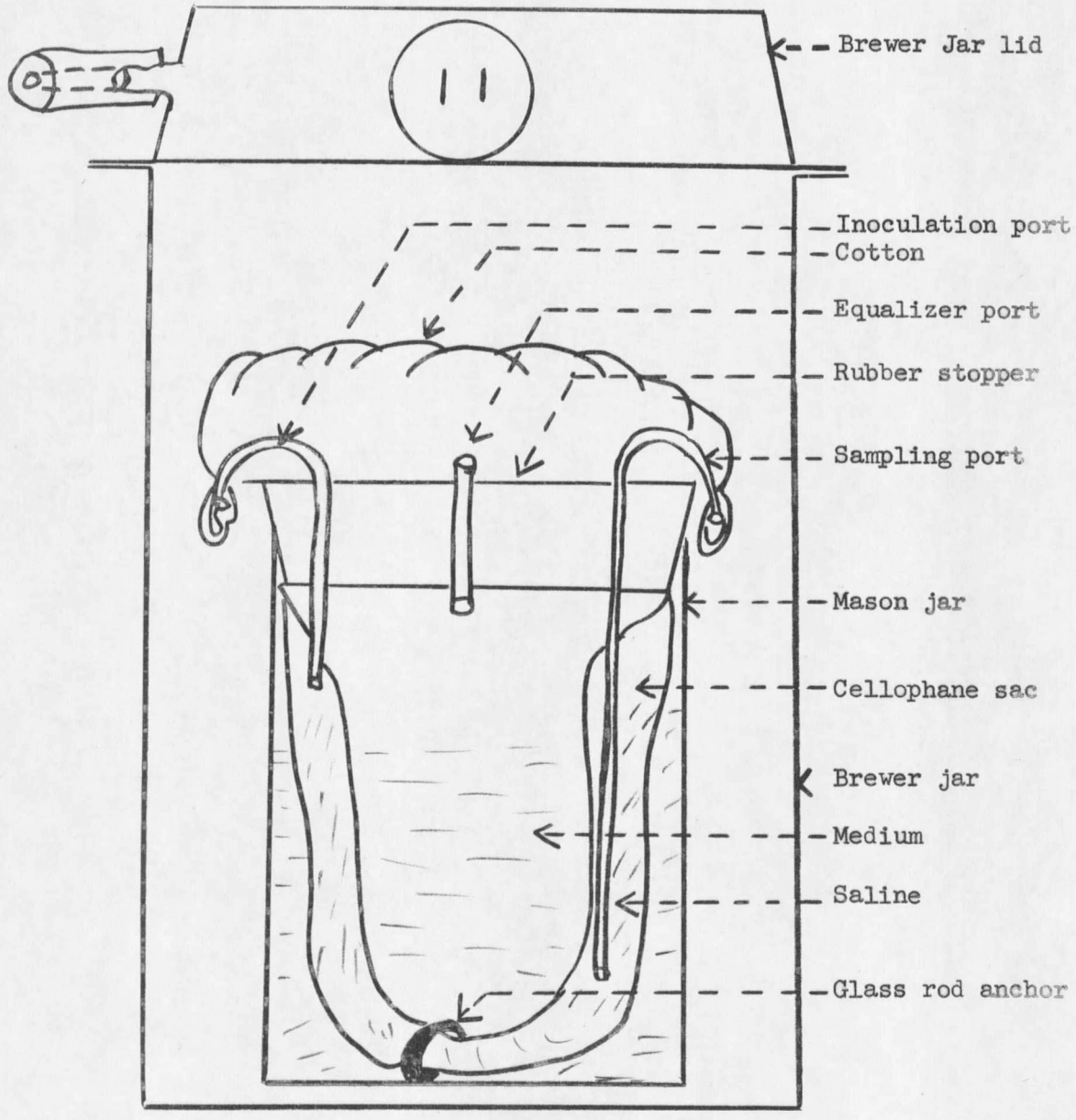


FIG. 1. Cellophane sac culture system.

Erlenmeyer flasks into which had been layered about ½ inch of cooked lean beef and a few small balls of steel wool. The pH in each bottle was adjusted to 7.4 with 5 N NaOH solution. An effort was made not to fill the flasks more than two-thirds full to avoid "boiling over" as pressure in the autoclave decreased. Autoclaving of these flasks of media was for 30 minutes at 121 C. In all instances, flasks of medium minus the meat were also prepared. These fluids were added to culture flasks immediately after autoclaving so that the culture vessels were filled almost to the top. The filled flasks were cooled in a water bath. Once the flasks cooled to approximately 37 C, they were inoculated with actively growing cultures of Strain 7170. Immediately after inoculation the toxin production flasks were sealed with vaspar (Hall, 1928) and incubated at 37 C in a water bath.

A "general toxin medium" had the following composition:

3% Trypticase (BBL 02-148)
0.5% Yeast extract (Difco 0127-01 or BBL 02-159)
0.001% $MgSO_4$
0.5% Na_2HPO_4
0.2% Soluble starch (Argo Div. Corn Products Co.)
0.1% glucose

The above ingredients were dissolved in distilled water and the solution was dispensed into Erlenmeyer flasks into which had been layered about ½ inch of cooked lean beef and a few balls of steel wool. The medium was autoclaved with the same care as was taken for the infusion medium.

Method for preparation and storage of toxic materials.

Fluids from cultures of Strain 7170 grown in the cellophane sacks

were pooled after the contents of various sacks were centrifuged for 30 minutes at 2520 x g. The fluids were dialyzed for one day against running cold tap water followed by further dialysis against frequent changes of cold distilled water for an additional 12 hours. Final dialysis was conducted against cold glycerol to diminish the volume to approximately one-half of the original. The glycerinated fluid in the sacks was placed in small screw-cap tubes in 3 ml volumes and stored at -20 C.

Toxic fluids of Strain 7170 grown in the infusion medium were centrifuged at 2520 x g for 30 minutes. The supernatant fluids were precipitated with 50% W/V (72% at 23 C) of ammonium sulphate. The insoluble material which rose to the top of the salted fluids was taken up with the aid of a stainless steel screen and was placed into a beaker. This material was dissolved in distilled water in an amount equal to 1/10 the original culture volume, and dialyzed (cellulose dialysis tubing, 1 1/8 inches inflated diameter, and 0.0010 inches thickness) against running cold tap water for 32 hours and then against frequent changes of cold distilled water for 24 hours. Lastly, dialysis against a number of changes of glycerol was done so that the final volume was about $\frac{1}{4}$ of the reconstituted $(NH^4)_2 SO_4$ precipitate.

Method for estimating optical density of proteins in acrylamide gels.

The relative optical density of proteins in polymerized acrylamide gels was estimated according to the instructions in the manual for use of the densiometer. The instrument used was a Chromoscan Model M61 (National Instrument Laboratory, Joyce Loebl Co. Ltd., England). This instrument was

run with a cam angle of 45°, a slit width of 0.25 mm and a gear ratio of 3 to 1. The beam of light was passed through a color filter #5038 and a neutral density filter #5031.

Method for the preparation and storage of egg yolk suspensions.

One yolk was used for each 500 ml of saline. The yolks were broken and thoroughly stirred with a glass rod. The egg yolk suspension was centrifuged at 5860 x g for 20 minutes and the supernatant fluid chilled and filtered through coarse filter paper to remove floating fat particles. Fifty ml quantities of the filtered suspension were placed in small plastic bottles and in three ml quantities in small screw-cap tubes. The partially-filled containers were stored at -20 C.

Thawed yolk suspensions that flocculated after incubating at 37 C for one hour followed by refrigeration for 12 hours at 8 C were considered unstable and were discarded. Usable suspensions were assayed for content of organic and inorganic phosphate.

Method for the preparation of lecithin.

Lecithin was prepared according to the method of Pangborn (1951). The jelly-like phospholipid was dissolved in 100 ml of ether and impurities in the form of a fine precipitate were removed by centrifugation at 365 x g at 4 C. The supernatant fluid was concentrated under vacuum and the lecithin was diluted in enough ethanol so that each ml of the alcoholic solution contained 500 ug phosphorus. This solution was stored at 5 - 8 C until used. Immediately prior to use, the alcohol in the solution was removed with the aid of vacuum and the lecithin was resuspended in a

solution of physiological saline.

Methods for obtaining and preparing erythrocyte suspensions.

Mouse erythrocytes were obtained from the hearts of anesthesized animals whose thoracic cavity was opened to expose the heart. The blood was collected in syringes which previously had been partially filled with 0.3 - 0.5 of 3% sodium citrate solution. A sharp 20 gauge needle was thrust into the right ventricle of the heart and about 1.5 - 2.0 ml of blood was removed from each mouse. The freshly drawn citrated blood of several mice was pooled and centrifuged at 650 x g for 10 minutes. The plasma and much of the buffy layer was removed with the aid of a Pasteur pipette connected to a water siphon. The packed erythrocytes were re-suspended in physiological saline solution and centrifuged as before. The supernatant layer of saline solution was removed with the Pasteur pipette and an effort was made to remove the remaining buffy layer of cells. The process was repeated a third time and the packed cells diluted with physiological saline solution to give 50% working suspension of erythrocytes.

Erythrocytes of chickens and guinea pigs were also obtained by cardiac puncture. The guinea pigs were anesthesized with ether but this was not necessary for the chickens. The guinea pig cells were treated in the same manner as the mouse cells while chicken cells were centrifuged at 164 x g because the cells are larger and, at the higher speed, would pack tight enough to result in partial hemolysis.

The erythrocytes from sheep and cattle were obtained by puncture of the jugular veins. Collection was made either into test tubes with 3%

citrate solution or into flasks containing glass beads or Zytel Nylon Resin (E.I. DuPont de Nemours Co., Wilmington, Del.) for defibrination. The cells were handled in the same manner as those of mice. The final suspension of sheep cells could not be made in borate buffered saline-peptone toxin diluent because these cells hemolyzed with the borate buffer. The cells were suspended in the saline-peptone diluent used for in vivo work.

In most instances, the cells were used immediately after the washing process, but at times, cells stored for a few hours had to be employed. Prior to use of stored cells they were washed, centrifuged, and resuspended at the desired concentration. Since the fagility of the cells increased with storage, they were discarded if not used within 24 hours after collection.

Method for the preparation of diluents for toxic materials.

For in vitro tests the diluent was a borate buffered saline-peptone preparation. For in vivo tests, the diluent was a saline-peptone solution adjusted to pH 7.0 - 7.4 with 1 N NaOH. The latter diluent was also used for hemolysis test in which sheep erythrocytes were used. Because phosphates interfere with lecithinase activity, the peptone used for the diluents was dialysed against running tap water for 32 hours and against frequent changes of distilled water for 24 hours. A 10% solution of Bacto-peptone (Difco 0118-01) was dialyzed, and after dialysis the peptone was tested for free phosphate by the method of Fiske-Subbarow (1925). The phosphate-free peptone solution was autoclaved for 30 minutes at 121 C. Enough of this dialysate was used in borate buffer so as to produce a

biuret reaction with an optical density of 0.20 at 550 mu using a Coleman #6 spectrophotometer and 10 X 75 mm cuvettes (Coleman 6-310A). This optical density corresponded to approximately 1% undialyzed peptone.

Toxin diluent was prepared in quantities of 300 ml with the following reagents:

75 ml boric acid (0.2M)
3.0 ml borax (0.05M)
36 ml of peptone soln.
188 ml saline (0.87%)

The pH of the toxin diluent with the above ingredients was adjusted to approximately 7.0 with either borax or boric acid solutions.

Diluent free of borate was prepared with 36 ml of dialyzed peptone and 264 ml of physiological saline. The pH of borate-free diluent solutions were adjusted to 7.0 - 7.2 with 1 N NaOH.

Before use, all batches of diluent were tested for hemolytic effects on mouse erythrocytes. The diluent solutions were kept under refrigeration for about a week before discarding.

Methods for estimating lecitho-vitellin (LV) reactions.

The LV tests were performed using either 1.0 ml or 0.2 ml quantities of toxin solution and egg yolk solutions. The tests were performed by mixing one ml of serial dilutions of the crude enzyme preparations with equal volumes of egg yolk suspension which contained 0.02 M calcium chloride. The mixtures were thoroughly mixed by shaking and incubated for 45 minutes at 37 C. Following the incubation period the test materials were refrigerated for eight hours at 6 - 8 C. The results were arbitrarily recorded as 1+, 2+, 3+, 4+, and - depending on the amount of turbidity

produced. The end point was considered as the tube containing the least amount of toxin which produced visible flocculation of the egg yolk.

When partially purified toxins were used, the tests were performed in a similar manner but the quantities of reactants were reduced to 0.2 ml of the enzyme and the same volume of egg yolk suspension. These mixtures were also incubated at 37 C for 45 minutes and then refrigerated for eight hours at 6 - 8 C.

In order to obtain a positive LV reaction with unusually weak toxin solutions it was often necessary to use previously frozen and thawed egg yolk suspensions that were somewhat unstable. In these instances great care was used in evaluating the reactions. Observations were made on at least 5 control tubes which had to be significantly different from reaction tubes containing purified toxin.

Method for estimating in vitro hemolysis.

Serial dilutions of crude and purified enzyme preparations were mixed with equal volumes of erythrocyte suspensions. Volumes of 0.2 ml of the reactants were used with partially purified toxins; while volumes of 1.0 ml of the substances were used in tests with crude toxins. Enough calcium chloride was added to the erythrocyte suspensions to yield 0.02 M. The mixtures were incubated at 37 C in a water bath for 45 minutes followed by refrigeration for eight to twelve hours. The amounts of hemolysis in each tube was estimated colorimetrically using either a Klett-Summerson colorimeter or a Coleman #6 spectrophotometer. For the colorimetric estimates it was necessary to dilute the test samples. The dilution necessarily

varied with the concentration and amount of erythrocytes used. For tests using 1.0 ml volumes of 5% erythrocyte suspensions with 1.0 ml volumes of toxin dilutions, it was necessary to add 6.0 ml of physiological saline solution to the reactants. This addition of saline permitted coverage of the whole spectrum of hemolysis in the scale of the Coleman #6 spectrophotometer. These estimates were made with 19 X 150 mm cuvettes (Coleman #6-304A) at 550 mu. For estimates with 0.2 ml amounts of erythrocytes, 1.0 ml of saline solution was added to the reactants. For the latter estimates 10 X 75 mm cuvettes (Coleman #6-310A) were used.

Samples for determination of in vivo hemolysis were withdrawn by cardiac puncture from inoculated animals in the moribund stage or shortly after death. Blood in 0.1 ml amounts was withdrawn from baby chickens or mice with a tuberculin syringe fitted with a 26 gauge needle and containing an equal volume of 3% sodium citrate. The blood was then transferred into test tubes containing 8.0 ml of normal physiological saline solution. The contents were carefully mixed, and the samples taken from mice were centrifuged for 15 minutes at 650 x g and those taken from chickens at 164 x g. The percentage of hemolysis was estimated colorimetrically as was described previously.

Method for determining liberated phosphorylcholine.

Phosphorylcholine liberated by the action of phospholipases on various subtrates was determined by analyzing for acid soluble phosphate. The initial step consisted of precipitating the sample with 10% trichloracetic acid. The acidified suspension was filtered and the filtrate was

digested by "wet ashing" with sulphuric acid and 30% hydrogen peroxide (Martland and Robinson, 1926). Assays for phosphate were conducted with the reducing reagent of Fiske Subbarow (1925). The blue color of the reactions was measured at 660 mu with either a Klett-Summerson colorimeter or a Coleman #6 spectrophotometer.

Methods for estimating toxicity and gross pathology.

Toxicity of inoculated mice and new-born chickens was estimated by the probits method of Finney (1952). The animals were inoculated intravenously or intramuscularly with serial two-fold dilutions of the toxins in 0.2 ml amounts. The intravenous inoculations were given to mice into the tail veins after the animals were placed beneath a 100 watt lamp for a few minutes to distend the vessels. After "warming", the animals were transferred one at a time to a plastic holding device where the body of the animal was held rigidly in place while leaving the tail exposed to the operator for the inoculation. The inoculations were made with 1.0 ml syringes fitted with sharp 26 gauge $\frac{1}{2}$ inch needles.

Intravenous inoculation of baby chickens was done by cardio-puncture with tuberculin syringes fitted with sharp 24 gauge one inch needles. Following the inoculations the animals were watched a few minutes for staggering or bloody frothing from the beaks which would indicate inoculation trauma. Traumatized animals were discarded and replaced with successfully inoculated chickens.

Intramuscular inoculations into mice were given in 0.1 and 0.2 ml amounts into the muscles of the hind legs. Baby chickens were inoculated

into the breast muscle. Inoculations into both species were made with a tuberculin syringe fitted with a 26 gauge needle.

Toxicity determinations of solutions of purified toxins were made by intradermal inoculation of mice. These inoculations were made in quantities of 0.05 ml with a tuberculin syringe and a sharp 27 gauge needle into the shaved skin of the abdominal area.

Necropsy examinations were conducted on chickens and mice according to the procedures described by Jones and Gleiser (1954). Procedure for chickens. The first step consisted of making external examinations for discharges from the eyes, nose, beak and cloaca. The appearance of the comb and legs were also noted. The animals were dipped in a solution of amphyl, 1:200 and the body cavities were exposed by pulling the legs laterally so as to break the skin and loosen the coxo-femoral joints. The skin and feathers were removed from the beak to the cloaca and from the sides of the neck and the body. The condition of the musculature and subcutaneous venation were recorded. The first instrumental cut was made through the abdominal wall across the anterior abdomen near the tip of the breast bone and parallel to the body length. The cut was extended along either side of the abdomen and forward along the rib articulations. The breast and other layers of tissue were removed from the ventral area to examine the abdominal and pleural cavities. The presence of fluids, exudates, blood and fat droplets were noted. Samples of heart blood were taken at this time for in vivo hemolysis determination. The visceral organs were removed and studied in comparison with normal structures. The heart and lungs were examined in situ and compared with normal organs. The air sacs

were examined for the presence of fluids and their appearance recorded. The esophagus and crop were examined by making an incision from the beak through the throat and down to the crop. The trachea was examined by making an incision along its length to the bronchi.

Procedure for mice. The first step consisted of making external examinations and dipping the animals in a 1:200 solution of Amphyl. The initial incision to observe the body cavities was a straight cut from the sternum to the symphysis. The animal was then turned to the opposite direction and the cut was extended anteriorly through the rib cage. The abdominal and pleural cavities were examined for presence of bloody fluids or bloody fluids containing fat droplets. Blood samples were taken from the heart before any of the organs were removed from the carcass. After sampling of the blood was completed, the organs of affected animals were removed one at a time and were compared with normal organs. Their size and appearance were recorded.

Method for determining proteinase activity.

The proteinase activity of culture fluids of Strain 7170 and of the electrophoretically purified toxins was studied by gelatin liquefaction and by chromatography of hydrolyzed X-ray film emulsion. The effects on gelatin were studied by mixing 0.5 ml quantities of gelatin solutions with 0.5 ml quantities of culture fluids or purified toxins. The mixtures were incubated at 37 C for eight hours. Control tubes contained gelatin with toxin preparations which were inactivated by heating at 95 C for 15 minutes. After the incubation period the tubes containing gelatin and

toxin solutions were immersed in cold water and observed for gelatin liquefaction.

Hydrolysis of X-ray film emulsion was studied by placing a drop of toxic material on a piece of undeveloped film. High humidity was attained by placing the test samples on test tube racks inside a water bath with a snugly fitting lid. The temperature of the water in the bath was regulated to 37 C. The heat necessary to maintain this temperature aided considerably in creating the very moist environment that was necessary to prevent evaporation of the test samples. Hydrolysis in the moist environment was conducted for a period of 12 hours at which time the pieces of X-ray film were removed one at a time. A drop of water was added to the same spot where the toxin had been placed. With the aid of a Pasteur pipette the drop of water was carefully mixed with the emulsion of the film. A small drop of the mixture was placed on silica gel H plates for chromatographic study. The plates were prepared with mechanical devices as outlined by Bobbitt (1963).

Once the spotted chromatography plates dried, two dimensional chromatography of ascending solvents was begun (Bobbitt, 1963). The first solvent was a mixture of 67 parts propanol and 33 parts of ammonium hydroxide. The second solvent was a mixture 3:1:1 butanol-acetic acid-water. The developed chromatoplates were visualized by spraying a 0.03% ninhydrin solution in 95% ethanol on the plates. After spraying, the plates were air dried and subsequently heated to 70 C for five minutes in an oven. The heated plates develop reddish spots at the location of amino acids or peptides.

Methods of estimating lipase activity.

Lipase activity of some of the partially purified toxins was estimated by a procedure incorporating the techniques of Sebald and Prevot (1960), and Willis (1960). To a sterilized 1% solution of purified agar (Difco, 0560-02) was added 1500 units penicillin and 1500 ug/ml streptomycin. The agar was maintained in the fluid state by keeping it in a water bath set at 50 C. The following lipids in 1.0 and 0.1 ml amounts were placed in a number of petri plates:

(a)	Butter oil,	0.1 ml
(b)	Milk cream,	1.0 ml
(c)	Egg yolk,	1.0 ml
(d)	Tween 20,	1.0 ml
(e)	Tween 65,	1.0 ml
(f)	Tween 85,	1.0 ml

The control plate had no substrate additives. The lipid contents of each plate were mixed with 20 ml of agar. After mixing, the agar was allowed to solidify by leaving the plates undisturbed for about 30 minutes at room temperature. Several wells were then cut in the agar of each of the plates and the holes were partially filled with solutions of crude toxin, partially purified toxin, heat inactivated crude toxin (95 C for 15 minutes) and heat inactivated purified toxin (95 C for 15 minutes).

The plates were transferred to a preheated moist chamber and were incubated for 40 hours at 37 C. At the end of this period the plates were removed from the moist environment and were flooded with a saturated solution of copper sulfate. This reaction was allowed to proceed for 20 minutes and the excess copper salt solution was decanted. The plates were placed in an incubator to dry for approximately 20 minutes at 35 C. After

drying they were refrigerated for five minutes to allow the formation of the greenish copper salt complexes with liberated fatty acids.

Serological procedures.

Development of standards. Four diagnostic antisera were standardized against a preparation of culture fluid of "7170-V". One of the sera was given a value of 600 units of antitoxin/ml, a unit of antitoxin being that amount of this serum which completely inhibited the LV reaction. On this basis it was estimated that this preparation of 7170-V contained 750 toxic units/ml. This preparation was used to standarize the remaining three sera using egg yolk as an indicator. The end-points were estimated as the highest dilution of sera which inhibited the yolk reaction of 10 LV units of toxin.

The neutralization tests were conducted as follows: To a volume of 1 ml of serum dilution containing 0.06 M/ml calcium chloride was added 1 ml of diluted toxin solution. The toxin-antitoxin mixture was held for 40 minutes at room temperature. After this incubation period 1 ml of a yolk suspension was added. The mixture was incubated at 37 C for 45 minutes and 5 C for approximately eight hours.

Serological tests with purified toxins. Because of the small amounts of toxins harvested from columns of disc gel electrophoresis, the reactants for these serological tests were reduced in volume to approximately 0.2 ml of each of the toxin solution, serum and test indicator. The tests were conducted in duplicate for the most part, but in a few instances they were conducted in triplicate or quadruplicate. Appropriate controls

for toxin, sera, diluents and indicators were always included. For hemolysis neutralization tests, the erythrocytes used were always freshly drawn and prepared. Antitoxins were sometimes diluted to desired levels in toxin diluents and frozen for future use.

Method of conducting disc gel electrophoresis.

Partially purified toxins of Strain 7170 were prepared with the electrophoresis technique described by Ornstein and Davis (1960). The potentiometer of the power supply was adjusted so that each get column conducted 0.16 watt. After approximately 2½ hours of operation a marker band was seen to have traveled about 50 mm. At this time the gel columns were removed from the tubes and were used either for developing protein patterns or were cut into slices which corresponded to the areas where the gels contained partially purified toxins.

Staining gel columns and developing "cutting patterns". Gel columns that were to be stained were put inside small test tubes containing enough 1% aniline blue black solution to cover the gel, and allowed to stain for one hour. The stain was removed from the test tube by decantation while the gel was retained in place with the aid of a metal screen. The test tube was then filled with 7% acetic acid and the decantation repeated. The rinsing of excess dye was repeated several times while the column slowly released the dye. Following the last rinse, the column was placed on a petri dish cover, covered with a few drops of destaining solution, and inserted into a destaining tube. Air bubbles in the sides of the tube were removed with the aid of a stiff wire and the bottom and tops

of the tube were filled with additional destaining solution.

The prepared destaining tubes were placed in the upper vessels of the electrophoresis vessel, and both of the vessels were filled with 7% acetic acid. The electrodes were connected to the power supply and a current of about one watt was applied to each tube. Electrophoretic removal of excess dye was considered complete when it was seen that the lowermost portion of the gel which did not have any sample material became clear. At this time the tubes were removed from the vessel, and the gels were dislodged with air pressure applied with a two ounce rubber bulb. Because the gel columns sometimes were expelled with considerable force, the outlets of the tubes were directed toward a beaker which was partially filled with 7% acetic acid. The liberated gel columns contained a number of dark stained bands or discs. These columns were placed in 10 X 100 mm test tubes which were filled with 7% acetic acid and closed with cork or rubber stoppers.

Once the columns were placed inside the small test tubes, the cutting patterns could be prepared by placing thin strips of adhesive tape along the length of the tubes and drawing on the tape the visible lines of proteinaceous material in the gel column. When the bands had been traced on the tape, it was removed from the test tubes and attached to the table top. Unstained gel columns were cut into a number of sections which corresponded to the bands of stained protein traced on the adhesive tape. Cutting of gels was done with sharp razor blades.

Collecting and preserving purified toxins. Proteinaceous bands cut from the same location of a number of gel columns were pooled in screw-cap test tubes, and were stored in a freezer until needed. To remove the proteinaceous material from the bands, it was necessary to add to the cut gels toxin diluent in an amount to correspond to 0.1 ml for each band. The toxins diffused away from the gels and into diluent for 8 to 12 hours.

EXPERIMENTAL RESULTS

SECTION I

EXPERIMENTS WITH CRUDE TOXIN SOLUTIONS

Identification and characteristics of C. hemolyticum 7170.

Morphological and cultural characteristics. Stained smears of an 18 hour culture of Strain 7170 growing in liver infusion medium revealed Gram-positive and Gram-negative rods. The organisms measured approximately 4.0 u in length by about 1.0 u in width. A few of the cells contained oval subterminal spores, and the sporangia were only slightly swollen.

Several freshly prepared blood agar plates were streaked with the culture material and quickly placed under anaerobiosis and incubated for 72 hours at 37 C. At the end of the incubation period, small grayish colonies could be seen on the agar. Some of the colonies were raised and smooth with entire edges while others were flat and somewhat rough with uneven edges. Microscopic observation of the colonies revealed organisms similar to those seen in slides made from broth cultures. The smooth colonies had zones of partial hemolysis extending about 3 mm beyond the colony.

Some tubes of infusion medium were inoculated, incubated for 18 hours, and a pool of the broth from these tubes was centrifuged at 12,100 x g for 30 minutes. The clear dark supernatant fluid was used for toxicity and serum neutralization tests. This supernatant fluid was lethal to both mice and baby chickens. Intravenous inoculations of 0.2 ml killed mice in three hours when it was diluted to 1:2 but not when diluted 1:10. The

undiluted culture fluid was completely neutralized by mixing it with an equal volume of C. hemolyticum antitoxin containing 900 units/ml. The toxin reacted with egg yolk suspension to produce typical LV reactions. Undiluted culture fluids also hemolyzed 5% suspension of both mouse and chicken erythrocytes. The mouse cells were completely hemolyzed within five minutes of mixing. The chicken erythrocytes hemolyzed after about 30 minutes of incubation at 37 C.

The biochemical tests for Strain 7170 included gelatin liquefaction, reaction in iron milk, reduction of nitrates, production of hydrogen sulfide, production of indole and fermentation of glucose, maltose, lactose, sucrose and salicin. A summary of the characteristics of this organism are given in Tables 1 and 2.

Studies on toxic culture fluids.

Toxin production in four media. After it was ascertained that the organism was a toxin-producing strain of C. hemolyticum, three small batches of 100 ml each of toxin solution were prepared using different media. A fourth batch was prepared using the cellophane sack culture system. The media were inoculated with 1 ml amounts of actively growing Strain 7170. With the exception of the cellophane sack cultures, two ml samples were taken from the cultures at 4, 8, 12 and 16 hours after inoculation. The samples were assayed for hemolysis, LV and lethality. The results are shown in Table 3 where it can be seen that toxin production began approximately four hours after inocultaion. Maximal toxic activity was seen after about 12 to 16 hours of incubation. Although digest medium

TABLE 1. Some characteristics of C. hemolyticum 7170 (Physiology)

Media*	Reaction
Gelatin	Liquefied in 5 days**
Iron milk	Soft coagulation
Nitrate medium	Nitrate not reduced
Tryptone medium	Indole produced
Glucose medium	Acidified
Maltose medium	Not acidified
Lactose medium	Not acidified
Sucrose medium	Not acidified
Salicin medium	Not acidified

* Gelatin; used Thiogel medium (B B L 01-293). Medium had 5% gelatin.
Iron milk; whole raw milk with a small ball of steel wool.
Nitrate medium; Stock culture medium with 0.1% KNO_3.
Tryptone medium; Ground beef stock culture medium with added 1% tryptone.
Carbohydrate media; Thioglycollate medium without dextrose (B B L 01-136C) to which was added the required carbohydrate in amounts of approximately 0.5%.

**All tubes incubated anaerobically in Brewer jars for five days.

TABLE 2. Some characteristics of C. hemolyticum 7170.
(Toxin neutralization)

Injection	Indicator	Effect
0.1 ml toxin + 0.1 ml saline	2 mice, IV	Both died
0.1 ml toxin + 0.1 ml saline	2 chickens, IV	Both died
0.1 ml toxin + 0.1 ml serum 1	2 mice, IV	Both lived
0.1 ml toxin + 0.1 ml serum 1	2 chickens, IV	Both lived
0.1 ml toxin + 0.1 ml serum 2	2 mice, IV	Both died
0.1 ml toxin + 0.1 ml serum 2	2 chickens IV	Both died
0.1 ml toxin + 0.1 ml saline	0.2 ml yolk	LV pos.
0.1 ml toxin + 0.1 ml serum 1	0.2 ml yolk	LV neg.
0.1 ml toxin + 0.1 ml serum 2	0.2 ml yolk	LV pos.

Serum 1 = C. hemolyticum serum No. 8053 (Veterinary Research Lab. MSC)
Serum 2 = C. perfringens type A serum No. K680 (Burroughs Wellcome Co.)

TABLE 3. C. hemolyticum toxin tests.

Identification	Medium	Incubation Time	Amt. Growth	LV	Hemo	LD* Chicks	Mice
Batch #1	General toxin medium	4	None	-	ND	ND	ND
		8	Slight	4	32	ND	ND
		12	Slight	8	64	ND	ND
		16	Slight	4	64	-	-
Batch #2	Liver Infusion	4	Slight	+2	ND	ND	ND
		8	Slight	4	16	ND	ND
		12	Slight	16	256	ND	ND
		16	Slight	16	128	-	+
Batch #3	Digest medium	4	Slight	16**	ND	ND	ND
		8	Moderate	128	512	ND	ND
		12	Heavy	128	2048	+	+
		16	Heavy	64	4096	+	+
	Cellophane sack	16	Very Heavy	128	2048	+	+

* Lethal dose. Inoculum 0.2 ml IV
** Medium control gave a reaction at 1:2 negative at 1:4
ND Not done
\+ Positive reaction
\- Negative reaction
Numerals Reciprocals of dilutions of culture fluids

was superior to the other media tested, uninoculated digested medium reacted with egg yolk to produce an atypical LV reaction. The possibility that contamination of the control medium was responsible for this reaction was ruled out when the medium was subjected to sterility tests.

Verification of purity. All cultures which were used for production of toxin were examined microscopically for gross contamination. Samples of fluids used in the production of toxins were also streaked on blood agar plates which were incubated aerobically and anaerobically. Fluids containing contaminants at the time of harvest were discarded. At the time of harvest and immediately following centrifugation of the organisms, the supernatant fluids were subjected to a serum neutralization test. All culture fluids used in the production of the raw toxins reacted with egg yolk to produce LV positive reactions that were completely neutralized by C. hemolyticum antitoxin.

Effect of pH on LV reaction. Since a number of factors affect the reaction rate of the C. hemolyticum toxin, it was thought necessary to first find out the effect of pH. Two-fold dilutions of toxin (Batch 4B) were made in toxin diluent (See page 22). The reactions of these were adjusted to the desired pH with 1 N NaOH and 1 N HCL prior to making the dilutions. To each of the dilution tubes an equal amount of egg yolk solution containing 0.026 M $CaCl_2$ was added. The pH of these egg yolk solutions had been adjusted to those of the toxin dilutions, to which they had been added. These were 5.0, 5.8, 6.7, and 8.0.

The results given in Table 4 show that C. hemolyticum toxin (Batch 4b) was active from pH 5.0 to 8.0. The range of activity may have extended

beyond the limits tested, but at pH 5.0 the egg yolk becomes somewhat unstable, and it could be seen that at pH 8.0 there was less activity than in some of the lower ranges tested. Atypical LV reactions with two layers of opalescence were observed in those tubes that contained most toxin. The reactions were particularly noticeable at pH 5.8, 6.7 and 8.0.

TABLE 4. Effect of pH on LV reaction (Batch 4B)

Final pH	Dilutions of crude toxin 1:3	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Yolk
5.0	3+	3+	3+	2+	+	w+	-	-	**
5.8	4+*	3+	3+	2+	+	w+	-	-	-
6.7	4+*	4+*	3+	3+	2+	+	-	-	-
8.0	3+*	3+*	2+	+	w+	-	-	-	-

* Tubes showing two layers of opalescence.
w+ Very weak reaction.
\+ Good reaction.
2+ Marked reaction.
3+ Strong reaction.
4+ Very strong reaction.
\- No reaction.
** Yolk somewhat unstable; formed opalescence after the refrigeration period when left at room temperature for two hours.

Effect of calcium ions on the LV reaction. Seven series of toxin dilutions containing different amounts of calcium chloride were tested for LV reactivity. The results are shown in Table 5 where it can be seen that the toxin reacted with egg yolk without the addition of calcium ions. It is also seen that the reaction end-points were increased with small

TABLE 5. Effect of calcium ions on LV reaction.

Amt. of Ca++	Dilutions of Toxic preparation 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Yolk Cont.
0.01	3+	3+	2+	2+	+	w+	-	-	-
0.005	3+	3+	2+	2+	+	w+	-	-	-
0.001	3+	3+	2+	2+	+	+	w+	-	-
0.00005	3+	3+	2+	2+	+	+	w+	-	-
0.001	3+	3+	2+	2+	+	+	w+	-	-
None	3+	2+	2+	+	-	-	-	-	-
0.0+EDTA	+	w+	-	-	-	-	-	-	-

4+	Very strong reaction
3+	Strong reaction
2+	Marked reaction
+	Good reaction; easily visible
w+	Weak reaction; difficult to see
-	Negative reaction

additions of the salt. The addition of ethylene diamino tetraacetic acid to the reactants markedly inhibited the reaction, but it was not completely inhibited.

Liberation of acid soluble phosphate from egg yolk. A standard phosphate curve was prepared as a reference with dilutions of a solution of 1 uM/ml of K_2HPO_4. All samples were placed in 19 X 150 mm cuvettes (Coleman No. 6-304A) and the amount of blue color developed with the Fiske Subbarow reducing reagent was estimated at 660 mu in either a Coleman Model 6A spectrophotometer or a Klett-Summerson 800-3 colorimeter.

The results with egg yolk are shown in Table 6. It can be seen that with high concentrations of toxin the results were about the same with both concentrations of calcium chloride. As the amount of toxin solution was reduced, the samples with 0.001M calcium chloride were LV positive to higher end-points than the samples with the higher concentration of calcium salt, but liberation of acid soluble phosphate was not proportional to the LV reactions. Slightly more phosphate was obtained with the samples containing the higher concentration of calcium.

Hemolysis pattern with chicken and mouse erythrocytes. Duplicate dilutions of C. hemolyticum culture fluids were made in peptone water placed in 1.0 ml amounts in 15 X 85 mm test tubes. To each of these tubes was added 1.0 ml of either 5% mouse erythrocytes or 5% chicken erythrocytes suspended in peptone saline containing 0.026 M calcium chloride. The toxin-erythrocyte mixtures were incubated at 37 C for 45 minutes and at 8C for about 12 hours. Appropriate controls for erythrocytes were included in each test. The results of two sets of dilutions were averaged and

TABLE 6. Liberation of phosphate from egg yolk.

Toxin dilution	Optical density	LV	$CaCl_2$	Toxin inorganic PO_4 uM	Total PO_4* uM	Liberation PO_4 uM
1:4	1.48	3+	0.01	1.23	2.32	1.09
1:8	1.18	2+	"	.61	1.76	1.14
1:16	.84	2+	"	.31	1.20	.89
1:32	.66	+	"	.16	.83	.67
1:64	.39	±	"	.08	.37	.29
1:128	.34	-	"	.04	.25	.21
1:256	.21	-	"	.02	.03	.01
1:4	1.48	3+	0.001	1.23	2.32	1.09
1:8	1.08	2+	"	.62	1.60	.98
1:16	.60	2+	"	.31	.74	.43
1:32	.43	+	"	.16	.44	.28
1:64	.32	+	"	.08	.22	.14
1:128	.27	-	"	.04	.12	.08
1:256	.28	-	"	.02	.04	.02

* PO_4 ≡ These figures do not include the inorganic phosphate contained in the yolk suspension (0.32 uM PO_4≡/ml).

plotted in Figures 2 and 3. Mouse erythrocytes were found to be more susceptible to the effect of the toxin than were chicken erythrocytes. These figures also show that the pattern of hemolysis obtained with mouse cells is linear while the one obtained with chicken cells is irregular.

Zero hour control dilutions and the titration materials above were tested for phosphate content after wet ashing. The results showed no significant difference in phosphate content between the zero hour controls and the titration materials. The determination of acid soluble phosphate was repeated with various dilutions of toxin on 20% suspensions of mouse and chicken erythrocytes. It was found that there was a detectable amount of phosphate liberation from the erythrocytes of mice but not with those of chicken. The results are shown in Table 7.

Lethality assays. Undiluted and serial two-fold dilutions of toxin preparations 4b and 5 were inoculated intramuscularly and intravenously into mice and baby chickens. Several animals were used for each dilution. Preliminary titrations were often conducted with two animals per dilution, to determine approximately the lethal range. The titrations were repeated with at least four or five animals for the same dilution. The results of the titrations with these two toxin preparations are given in Table 8. An analysis of the data by the technique of probits (Finney, 1952) showed that the chicken lethality by intravenous inoculation was 1:2.4 with Batch 4b; that it was 1:121 with Batch 5. Intramuscular inoculations showed a titer of 1:1.4 with Batch 4b and 1:22 with Batch 5. The intravenous inoculations in mice showed titers of 1:3.5 with Batch 4b and 1:56 with Batch 5. Intramuscular inoculations showed a titer of 1:1 for Batch 4b

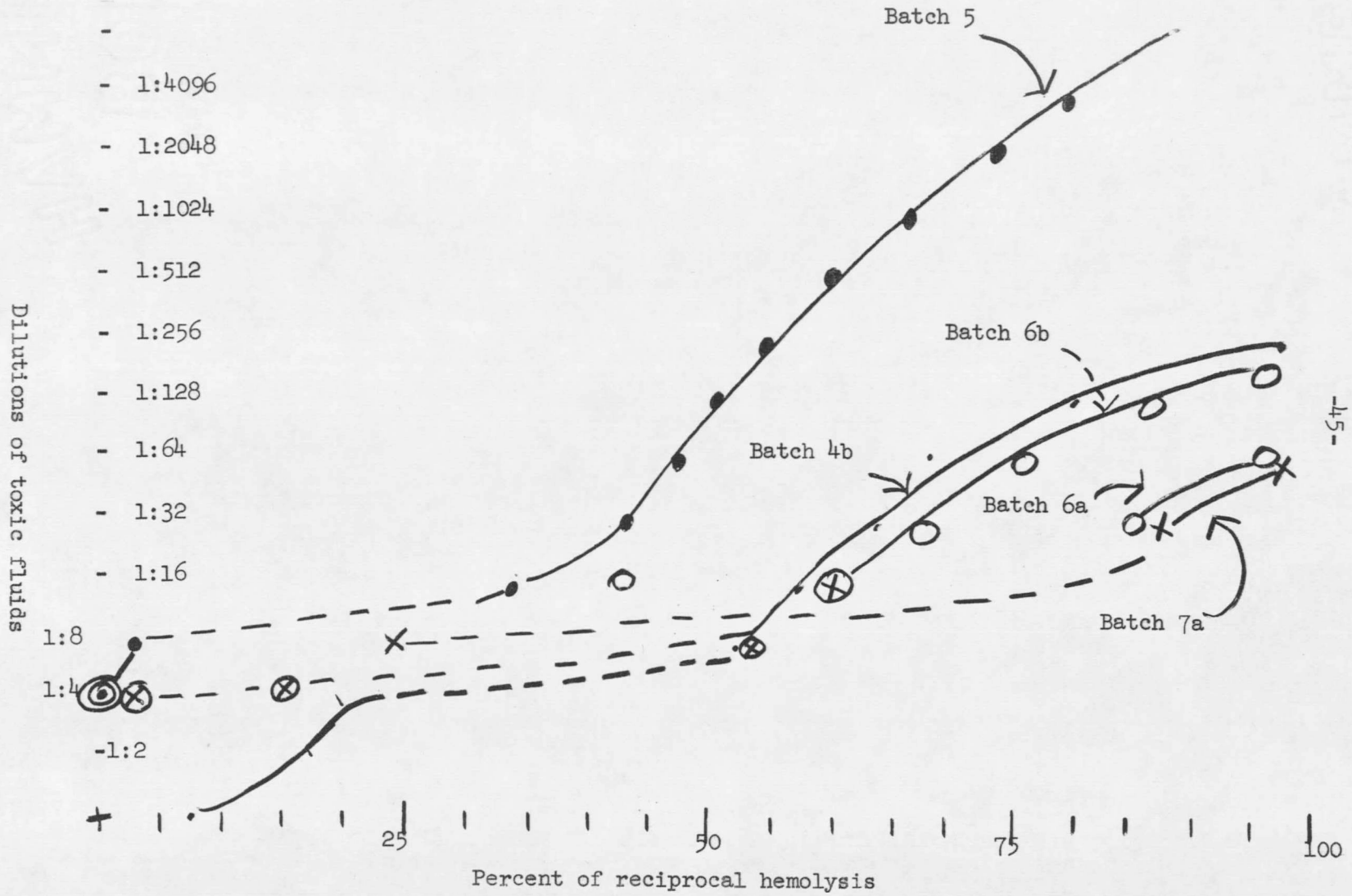


FIG. 2. Patterns of hemolysis on chicken erythrocytes with five different C. hemolyticum toxic fluids.

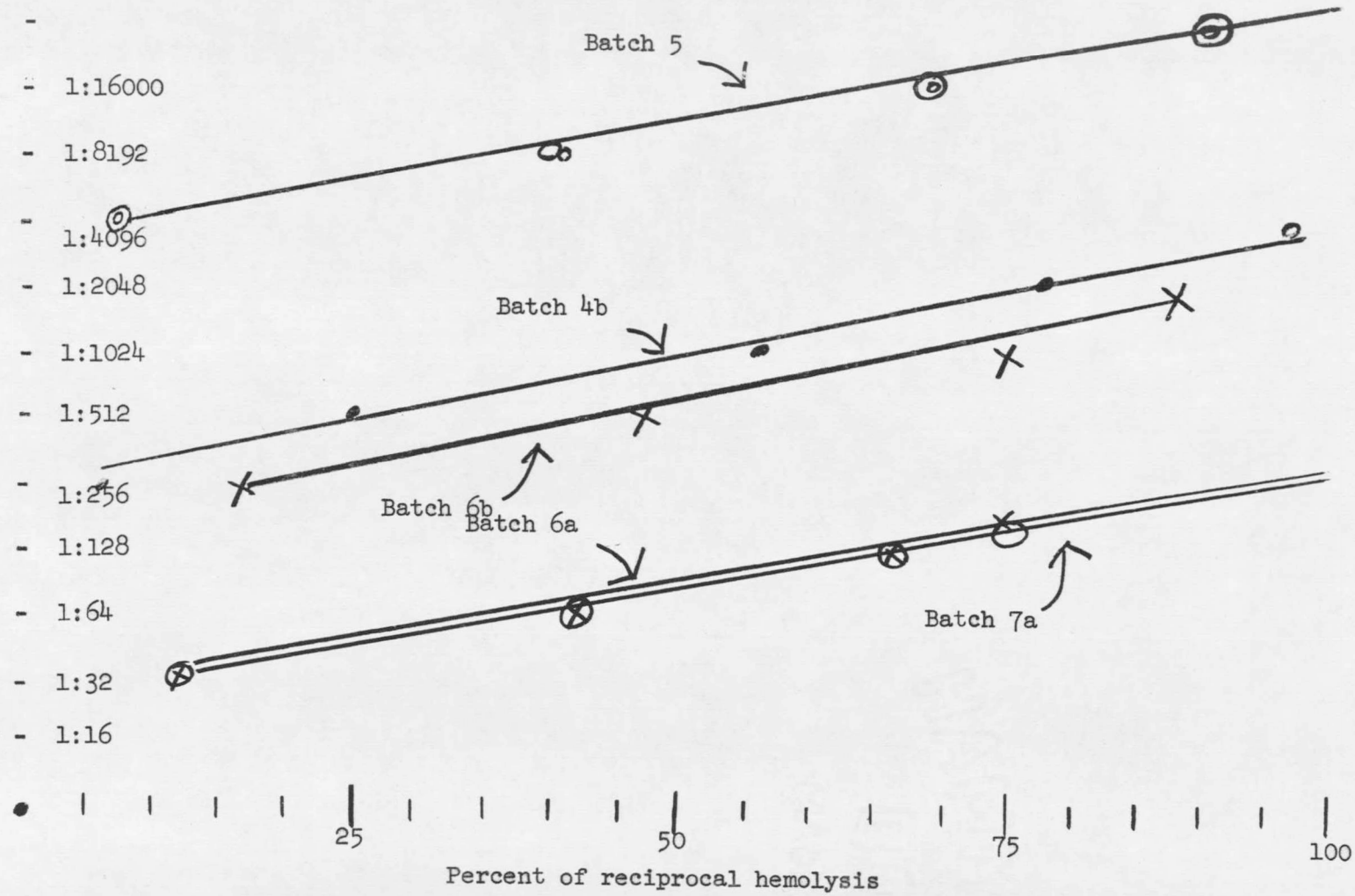


FIG. 3. Patterns of hemolysis on mouse erythrocytes with five different C. hemolyticum toxic fluids.

TABLE 7. Hemolysis and liberation of phosphate from erythrocytes by C. hemolyticum toxic fluid.

Toxin dilution	Chicken cells		Mouse cells	
	% Hemolysis	PO^{+}_{4} Liberation	% Hemolysis	PO^{+}_{4} Liberation
1:2	90*	0	90*	N.D.
1:4	90	0	90	N.D.
1:8	80	0	90	N.D.
1:16	70	0	90	1.17
1:32	35	0	90	0.69
1:64	15	0	90	0.36
1:128	T	ND	90	0.36
1:256	0	ND	80	0.08
1:1024	ND	ND	40	0.00
1:4096	ND	ND	10	0.00

* Percentage of hemolysis was estimated visually
ND Not done

The amount of phosphate is expressed in uM/ml.

TABLE 8. Titrations of two culture fluids in chicken and mice.

Dilution of toxin	Chicken inoculations				Mouse inoculations			
	Toxin 4b		Toxin 5		Toxin 4b		Toxin 5	
	IV	IM	IV	IM	IV	IM	IV	IM
Und.	6/6	4/6	-	-	6/6	3/6	-	-
1:2	3/4	1/4	-	-	5/6	0/6	-	-
1:4	0/4	0/4	-	5/5	2/6	0/6	-	-
1:8	-	-	5/5	9/10	0/6	0/2	5/5	4/5
1:16	-	-	5/5	7/10	0/2	0/2	9/10	1/5
1:32	-	-	10/10	2/10	-	-	9/10	1/5
1:64	-	-	10/10	0/10	-	-	3/10	0/5
1:128	-	-	0/5	0/5	-	-	0/10	0/5

Numerators = Dead animals; denominators = animals inoculated;
- = No inoculations at this dilution.

and 1:23 for Batch 5.

Gross pathology of inoculated animals. The inoculated chickens and mice showed similar symptoms after inoculations with C. hemolyticum toxins. Intravenous inoculations in mice usually resulted in extravasation of hemoglobin-stained plasma with fat droplets into the abdominal cavity. The lungs and bladder filled with hemolyzed blood, and shortly before death the animals had no more than about 15% of their erythrocytes in circulation. Intramuscular inoculations resulted in pathological changes similar to that brought about by intravenous inoculations; but in addition, these animals also showed affected muscles at the site of inoculation. The muscles were hemorrhagic and surrounded by an orange edema that contained fat droplets.

Baby chickens given intravenous injections of concentrated culture fluids showed extravasation of hemoglobin-stained fluids into the abdomen. The animlas would "bleed" from body openings, and shortly before death had few erythrocytes left in circulation. With moderate doses of toxin, the animals lived about 15 to 20 hours after injection, and multiple hemorrhagic foci were seen throughout the livers. Intramuscular inoculation resulted in alterations similar to those caused by the intravenous inoculation. These chickens also developed extensive edematous lesions at the site of inoculation.

Intramuscularly inoculated mice and chickens did not show signs of intravascular hemolysis until the animals were moribund. At this time it was possible to detect some hemolysis in the circulating blood.

Effects of aging and concentration procedures. Since some unexplained observations were made in the early experiments (See Table 4) and since there was no correlation between hemolysis and lethality, four additional small batches of toxic fluids were prepared to study the effects of aging and the method of concentration on the relationship between hemolysis and LV reaction. Two of these batches were prepared with cultures that were incubated for 16 hours and two were prepared with cultures that were incubated for 39 hours at 37 C. One each of the young and old culture harvests was concentrated by dialysis against glycerol. One each of the young and old culture fluids were concentrated by "salting out" with ammonium sulfate. The results are summarized in Tables 9 and 10 where it is seen that aging did not alter the ratio of reactions (Table 9), while the method of concentration did (Table 10).

Brief summary of results with toxic culture fluids.

In the first part of the present study it was shown that culture fluids of C. hemolyticum liberated phosphorylcholine from egg yolk suspensions and from the erythrocytes of mice but not from those of chickens. The hemolysis patterns with mouse cells were essentially linear while those with chicken erythrocytes were irregular and characterized by two slopes of hemolysis with a flat plateau between these reactions. It was also shown that mouse erythrocytes were much more susceptible to hemolysis than the erythrocytes of chicken, but that the two species were about equally susceptible to the effects of the toxic substances. The LV to hemolysis ratios were not constant for a number of toxic preparations.

TABLE 9. Effect of culture aging on toxin harvest.

Toxin preparation 7170-VIa*		Toxin preparation 7170-VIIa	"Young to Old" ratio
LV	16	16	1:1
CH	28	29	1:1
MH	82	84	1:1
Toxin preparation 7170-VIb		**Toxin preparation 7170-VIIb**	**"Young to Old" ratio**
LV**	128	64	2:1
CH	35	34	1:1
MH	600	321	2:1

* Toxin preparation 7170-VIa = From young culture; glycerol concentrate
Toxin preparation 7170-VIb = From young culture; NH^4SO^4 pptd.
Toxin preparation 7170-VIIa = From old culture; glycerol concentrate.
Toxin preparation 7170-VIIb = From old culture; NH^4SO^4 pptd.

**LV = Lecithovitellin reaction; average of two tests.
CH = Chicken hemolysis end-points; average of two tests.
MH = Mouse hemolysis end-points; average of two tests.

TABLE 10. Effect of concentration procedure on toxin activity.

Toxin preparation 7170-VIa*		Toxin preparation 7170-VIb	Glycerol-NH^4SO^4 ratio
LV**	16	128	1:8
CH	28	35	1:1
MH	82	600	1:8
Toxin preparation 7170-VIIa		**Toxin preparation 7170-VIIb**	**Glycerol-NH^4SO^4 ratio**
LV	16	64	1:4
CH	29	34	1:1
MH	84	321	1:4

* Toxin preparation 7170-VIa = From young culture; glycerol concentrate.
Toxin preparation 7170-VIb = From young culture; NH^4SO^4 pptd.
Toxin preparation 7170-VIIa = From old culture; glycerol concentrate.
Toxin preparation 7170-VIIb = From old culture; NH^4SO^4 pptd.

** LV = Lecithovitellin reaction; average of two tests.
CH = Chicken hemolysis end-points; average of two tests.
MH = Mouse hemolysis end-points; average of two tests.

The above observations indicated that C. hemolyticum produced more than one substance which affected the LV-hemolysis-lethality ratios, and it was decided to isolate and characterize some of the substances in the culture fluids. Techniques used for the isolation and characterization of four hemolytic substances comprise the second part of this thesis which is discussed in the subsequent pages.

EXPERIMENTAL RESULTS

SECTION II

EXPERIMENTS WITH PARTIALLY PURIFIED TOXINS

Fractioning of toxic culture fluids.

A crude toxin solution of C. hemolyticum was fractioned into a number of components because it was suspected that culture fluids of strain 7170 contained more than one toxin. The suspicion was based on the observation that (1) the hemolysis patterns of chicken erythrocytes were irregular; (2) there was a lack of proportionality between LV reaction and hemolysis with some of the toxins; (3) there was no correlation between hemolysis and lethality; (4) some of the LV reactions developed two layers of opalescence.

The most potent culture fluid was fractioned with the disc gel electrophoresis technique of Ornstein and Davis (1960). To obtain isolation of proteins in the acylamide gel these authors suggested that no more than about 200 ug of protein be used per column. Toxin preparation 7170-V was tested for protein content by the method of Lowry (1960), and it was found to contain about 230 ug protein per 0.025 ml. The initial electrophoretic preparations were conducted using 100 ug protein per gel column to determine the number of protein constituents preparation 7170-V contained and to see if "biological activity" could be detected in the isolated materials. The term "biological activity" will be used to refer to hemolysis, egg yolk reaction or other tests.

Culture fluids of C. hemolyticum contained a fast moving protein of yellow color that could easily be discerned in the gel tubes while the

fractioning was in progress. No biological activity could be detected in this band and it was used as a "marker" for later experiments because with it, the relative positions of the toxic fractions in the gel columns could be determined. In the experiments using 100 ug of protein per column, the marker band was seen moving down the length of the tubes, and when it had traveled 30 mm the glass tube was taken out of the electrophoresis vessel, and the gel was decolorized as described on page 36.

The finished columns showed that the culture fluids contained about 12 protein constituents. Control gels were prepared with uninoculated medium. Gels prepared with the medium showed that there were five protein constituents. The constituents shared by both the toxic fluids and the medium were the faster moving non-hemolytic entities. From the preliminary experiments it was also learned that the bands of protein seen in the gel columns were too close to one another for suitable separation by simple cutting of the gel column.

Additional 30 mm columns were prepared for verification of the previous findings and to determine which areas of the columns contained protein bands with hemolytic or LV activity. It was found that all the biological activity was present in the slower moving proteins in the gel column. The medium control bands contained no hemolytic or LV reactive material. Three bands showed hemolytic activity; only one of these reacted with egg yolk suspension.

The preceding experiments showed that the culture fluids of C. hemolyticum could be fractioned into a number of protein constituents and that the 30 mm columns lacked definition and reliability because the

isolated bands were very close to one another. Furthermore, hemolytic and LV substances were present in the slower moving proteins. To better identify the isolated substances and to increase the definition, the amount of protein per gel column was increased to 200 ug and the length of the columns was increased to 60 mm. In these gels the yellow marker was allowed to travel 50 mm before the gels were removed from the electrophoresis vessels.

It was found that the 50 mm columns produced good patterns with which to work because the spacing between proteins was better than the one seen in the 30 mm columns. Drawings of 50 mm gel patterns of medium control and toxin preparation 7170-V are shown in Figure 4.

A number of 50 mm columns were prepared and as many of the bands as could be isolated by cutting were tested for hemolytic and LV activity. It was found that of 12 distinct protein bands, five produced hemolysis of mouse cells, and two of these reacted with egg yolk with typical LV reactions. Two of the other bands reacted with the yolk suspensions with atypical LV reactions, and the fifth isolated band was obtained in such small quantities that it did not react with egg yolk and was only slightly hemolytic.

The above experiment was repeated numerous times to verify the observations. At times, inconsistent patterns of hemolysis and LV were seen. It was assumed that the inconsistencies were due to cutting improper areas of protein contained in the gels. Because of these observations, all further work was based on selected partially purified toxins. These toxins were sampled for hemolysis and LV patterns four hours after

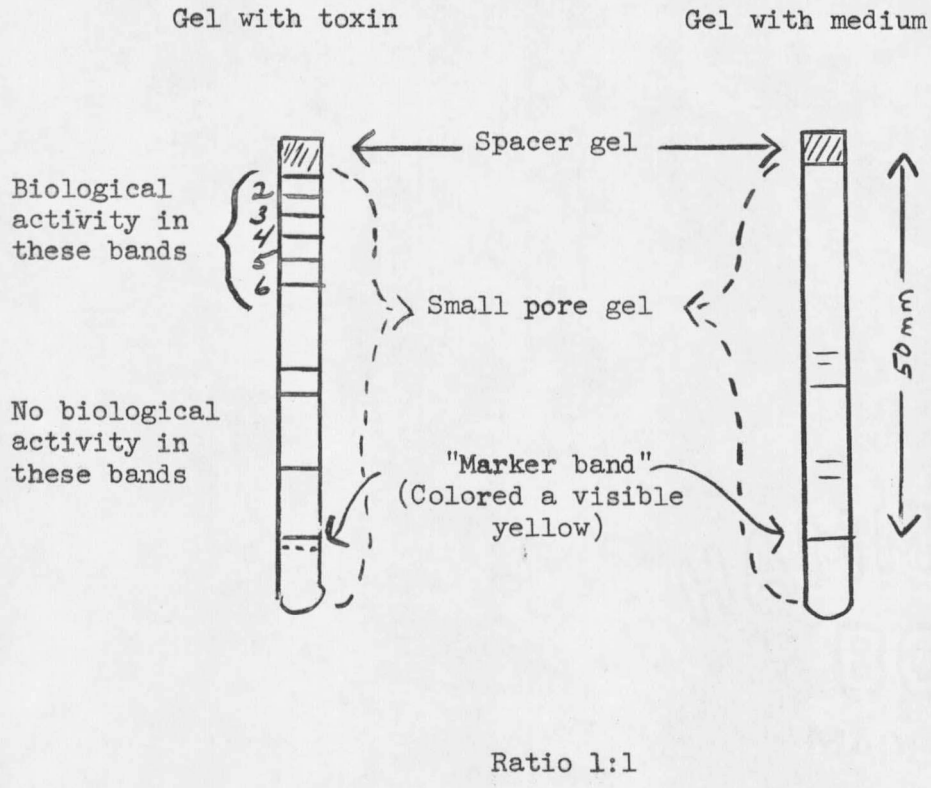


FIG. 4. Drawings of acrylamide gel columns of toxic fluid 7170-V and medium control.

diffusion was started. Samples producing atypical patterns were discarded. A densiometer pattern of normal protein distributions in a 50 mm gel column is shown in Figure 5. In this figure the hemolytic peaks are identified by numerals which will be used as reference items for the rest of the thesis. The relative concentrations of the various proteins are obtained from the peaks in the densiometer diagram.

Biochemical characteristics of isolated hemolysins.

Heat inactivation studies. Each of the protein band diffusates was divided into nine portions; one was heated at 65 C for five minutes; a second was heated at the same temperature for 10 minutes; a third was heated at 65 C for five minutes but to the sample was added a small amount of calcium chloride; a fourth was heated at 65 C for ten minutes with added calcium chloride; a fifth at 95 C for five minutes; a sixth at 95 C for ten minutes; a seventh at 95 C for five minutes with added calcium chloride; an eighth at 95 C for ten minutes with added calcium salt; and the ninth was saved as non-heated control.

All the isolated toxins could be inactivated by heat, but some of the isolated substances were more susceptible to the heat treatment than the others. The results are summarized in Table 11.

Serologic studies of purified toxins. To ascertain the purity of the isolated substances, they were studied serologically by gel precipitin (Lazear et al., 1958) and by serum neutralization of hemolysis. Two experiments were carried out with the gel precipitation technique using various concentrations of hemologous and heterologous sera with two

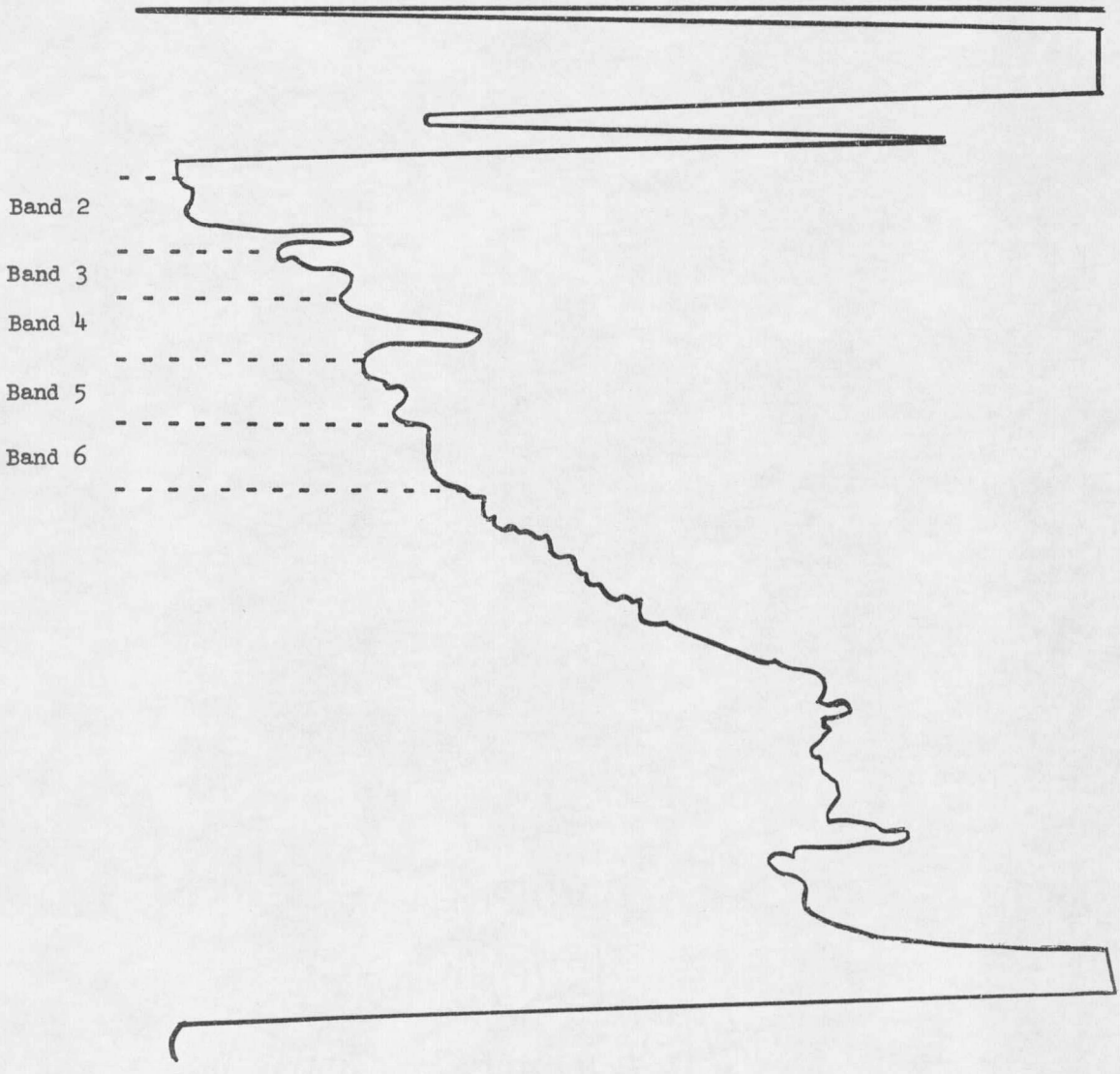


FIG. 5. Relative optical density of C. hemolyticum proteins in an acrylamide gel column.

TABLE 11. Heat inactivation studies of partially purified toxins of C. hemolyticum.

Temperature	Ca++	Time	Bands #2	#3	#4	#5
60 C	-	5 min.	38*	0	15	11
60 C	+	5 min.	14	0	7	2
95 C	-	5 min.	8	0	24	9
95 C	+	5 min.	7	0	32	4
60 C	-	10 min.	20	0	30	20
60 C	+	10 min.	25	2	22	20
95 C	-	10 min.	50	20	64	80
95 C	+	10 min.	98	76	86	92
95 C	+	15 min.	100	96	100	100

* The numbers refer to percentage loss in activity.

dilutions of partially purified toxins, but no results were obtained because antigen-antibody interface lines did not develop.

The first step in serum neutralization experiments was to standardize toxic preparation 7170-V against a homologous antitoxin which was given an arbitrary value of 600 units per ml. On this basis it was estimated that preparation 7170-V contained 750 toxin units per ml. This toxin then became a secondary standard with which a second C. hemolyticum antiserum was found to contain 2880 antitoxin units per ml. Two Clostridium novyi type B antisera were found to contain 39 and 48 C. hemolyticum toxin neutralizing units per ml.

The results with the serum neutralization work of the purified toxins are shown in Table 12 where it can be seen that the isolated hemotoxins could be neutralized by homologous and heterologous antitoxins. The table also shows that the hemolysins contained in the first three bands are serologically similar while the one contained in the fastest moving protein was serologically different from the others.

Hydrolysis of specific substances. The initial studies to characterize the four isolated hemotoxins provided a number of clues for future work. First, it was observed that the toxins reacted differently with egg yolk solutions. Two of the isolated substances produced LV patterns similar to those produced by lecithinase C; ie., opalescence with a layer of fat. This reaction suggested that two of the substances could be phospholipases. Second, it was observed that the stroma of acid laked erythrocytes were dissolved by the slowest moving of the hemotoxins. Third, it was observed that the hemotoxin that moved fastest was serologically different from the

TABLE 12. Summary of serological studies.

Band #	Serum*	Serum needed for neutralization	Toxin
2	1	2 units	(proteinase)
	2	2 units	
	3	4 units	
	4	4.5 units	
3	1	6 units	(Necrotic Hot-cold phosphdlipase)
	2	3.5 units	
	3	9 units	
	4	8 units	
4	1	3 units	(Weak Hot-cold phospholipase)
	2	1 unit	
	3	5.5 units	
	4	4 units	
5	1	3.5 units	(Lipase)
	2	1.0 unit	
	3	2 units	
	4	0.15 unit	

* 1 = C. hemolyticum antitoxin 8053; 600 units/ml
2 = C. hemolyticum antitoxin 5528; 2880 units/ml
3 = C. novyi type B antitoxin 3735; 39 units/ml
4 = C. novyi type B antitoxin 2492; 48 units/ml

slower moving entities. The fast moving substance reacted with egg yolk to produce atypical LV reactions; ie., opalescence without a fat curd. These observations, in conjunction with the work of Macfarlane (1950), suggested that it might be lipase.

Liberation of phosphorylcholine from phospholipids. Egg yolk suspensions treated with the isolated hemotoxins were tested for release of phosphorylcholine. Those toxins causing typical LV reactions liberated the chemical, while the substances not causing typical LV reactions did not. The toxins liberating phosphorylcholine are, by definition, phospholipases C.

The question arose whether the atypical reactions seen with two of the hemotoxins could be due to phospholipases which were not present in concentration sufficient to produce typical LV reactions. An attempt to solve the problem was made by preparing serial two-fold dilutions of the four hemotoxins and mixing these dilutions with egg yolk. The resultant reactions were assayed for phosphorylcholine. The results for this experiment are shown in Table 13. It was seen that it was not possible to rule out the possibility of band contamination or of phospholipase spread throughout the length of the gel columns.

A test with purified phospholipids was conducted. Egg yolk lecithin (prepared by the method of Pangborn, 1951) and commercial sphingomyelin (Nutritional Biochemicals Corporation, Cleveland, Ohio) were used. The phospholipids were suspended in physiological saline solution saturated with chloroform. The suspensions contained 50 ug of phosphorus per sample. The results of the hydrolysis of these substrates are shown in Table 14

TABLE 13. Liberation of acid soluble phosphate from egg yolk with partially purified toxins of C. hemolyticum.

Band No.	Type LV	Dilution	PO^4 Liberation
2	Atypical	Concentration	None
3	Typical	1:2	Positive
3	Typical	1:4	Positive
3	Typical	1:8	Neg. or trace
4	Typical	1:2	Positive
4	Typical	1:4	Positive
4	Typical	1:8	Neg. or trace
5	Atypical	Undiluted	Negative
6	None	Undiluted	Negative

TABLE 14. Effect of partially purified toxins on lecithin and sphingomyelin.

Substrate used	Incubation time (minutes)	Bands used			
		#2	#3	#4	#5
Lecithin	0	0*	0	0	0
Lecithin	45	0	23	17	0
Sphingomyelin	0	0	0	0	0
Sphingomyelin	45	0	6	9	0

* The numbers represent averages of liberated phosphorus in micrograms.

where it can be seen that approximately 50% of the lecithin was hydrolyzed but that a much smaller amount of sphingomyelin was attacked. The results also show that only bands 3 and 4 were active in hydrolyzing the phospholipids but with the results given it would be difficult to differentiate either of the phospholipases into either a lecithinase or a sphingomyelinase.

Effects of cysteine. To test for the presence of disulfide groups in the isolated hemotoxins, they were treated with cysteine which would disrupt the bonding and possibly inactivate the hemotoxin. Dilutions of 1:4, 1:8, and 1:16 of each hemotoxin were treated with 0.00 M, 0.02 M, 0.04 M and 0.06 M concentrations of freshly neutralized cysteine. The mixtures were allowed to react for 10 minutes. At the end of this time the treated materials and their controls were mixed with mouse erythrocytes. The results indicated that three of these hemolysins were susceptible to the action of the cysteine while one was not. The results are summarized in Table 15. These results showed that band #3 was not affected by the cysteine. It may have been that this hemotoxin was present in a greater concentration than the rest of the toxins. A second experiment was conducted with band #3 using higher dilutions of it than were used in the first test. Under these conditions it was found that band #3 is also susceptible to the effect of the cysteine.

Effect of EDTA. Because some of the bands reacted with egg yolk solutions and since it is known that the lecithinases C require calcium ions the four hemotoxins were treated with ethylene diamino tetraacetic acid (EDTA) to see if the chelating compound would effectively bind

TABLE 15. Cysteine inactivation studies.

Toxin dilution	Amount cysteine	Bands tested* #2	#3	#4	#5	Controls Cysteine	Gel
1:4	None	+	+	+	+	ND	0
1:4	.02 M	-	+	-	-	0**	0
1:4	.04 M	+	+	-	+	0**	0
1:4	.06 M	-	+	-	-	0**	0
1:8	None	+	+	+	90	As above	
1:8	.02 M	-	+	-	-		
1:8	.04 M	-	+	-	-		
1:8	.06 M	-	+	-	-		
1:16	None	90	+	+	20	As above	
1:16	.02 M	-	+	-	-		
1:16	.04 M	-	+	-	-		
1:16	.06 M	-	+	-	-		

*
+ = 100% hemolysis
- = 0% hemolysis
Numbers = Estimated % hemolysis at the given dilution
ND = Not done

** The erythrocytes darkened but did not hemolyze.

available calcium ions and inhibit the hemolytic effects. In this experiment the amount of calcium was adjusted to obtain a solution containing 2.4×10^{-3} uM/ml. Ethylene diamino tetraacetic acid was added to obtain 2.7×10^{-3} uM/ml. This amount of sequestering agent should be sufficient to bind the added calcium ions (Deverell, 1957). The results are summarized in Table 16 where it can be seen that all the isolated hemolysins were affected by the chelating agent. It should be of interest to note that band #3 hemolyzed prior to chilling and that it was only partially inhibited by EDTA. This would probably indicate once again that the toxin was present in very large amounts in proportion to the other hemotoxins or that the substance was contaminated with materials other than lecithinase C. Band #4 also reacted with erythrocytes, but did so only after the samples were chilled following incubation at 37 C.

pH optima. The pH studies were carried out with diffusates of gels adjusted to final reactions of pH 6.0, 7.0 and 8.0. The erythrocyte suspensions were also adjusted to the same reactions prior to mixing. It was found that the erythrocytes of mice hemolyzed at pH 6.0; therefore hemolysis at this hydrogen ion concentration could not be determined. With the remaining diffusate solutions it was shown that the hemolysin isolated from band #5 reacted more at pH 8.0 than at pH 7.0. The other hemolysins, bands 2, 3 and 4 were more reactive at neutrality than at pH 8.0. These results are summarized in Table 17.

While conducting these experiments it was seen that the hemolysin obtained from band #2 digested the acid hemolyzed stroma of erythrocytes. The other bands did not do so. This observation led to the belief that the

TABLE 16. Calcium requirement of C. hemolyticum toxins.

Band #	RBC	RBC+EDTA	LV	LV+EDTA	Controls
2	+	-	-	-	RBC=OK
3	+	70	+	-	RBC+EDTA=OK
4	+	*	+	-	Yolk = OK
5	+	-	+	-	Gel Diff. plus all above = OK
6	+	-	-	-	

+ = LV positive reaction or 100% hemolysis
- = LV negative reaction or 0% hemolysis
* = Complete inhibition at 37 C, but only partial at end of refrigeration
Numbers = estimated % reaction

TABLE 17. pH studies of partially purified toxins of C. hemolyticum.

	pH 7.0					pH 8.0				
Band #	U	1:2	1:4	1:8	1:16	U	1:2	1:4	1:8	1:16
2	+	+	80	40	-	+	+	+	60	20
3	+	+	+	+	+	+	+	+	+	60
4	+	+	+	90	70	+	+	50	10	-
5	+	70	10	-	-	+	+	50	10	-
6	80	30	T	-	-	20	T	-	-	-

+ = 100% hemolysis
- = no hemolysis
T = trace
Numbers = estimated % hemolysis
U = undiluted toxin

(Hemolysis at pH 6.0 could not be determined because of acid hemolysis.)

hemolysin in band #2 could be a proteinase.

Hemolytic range of isolated enzymes. As a further test to differentiate and characterize the isolated substances, their hemolytic range for erythrocytes of various species was studied. Cells of mice, chicken, guinea pig, cattle and sheep were used. Five percent suspensions of these erythrocytes were prepared. To the suspensions was added 0.02 M calcium chloride. The erythrocyte suspensions were mixed with equal volumes of isolated toxins. The mouse cells were the most susceptible to hemolysis by any of the hemotoxins. Chicken cells were the least susceptible. The chicken cells were not attacked by two of the isolated hemolysins, and those of cattle and sheep were not attacked by one of the hemotoxins. Variations in the degree of attack of the various cells was also seen. The results of these experiments are summarized in Tables 18 and 19.

Identification of a proteinase. The slowest moving of the electrophoretically purified hemotoxins, band #2, digested the stroma of acid hemolyzed erythrocytes. Because of this observation the four isolated hemotoxins were tested for proteinase activity. The first experiment was conducted with gelatin. The procedure for the test was previously described. The results showed that band 2 was the only one which contained substances affecting the gelation time of gelatin.

In order to verify the presence of the proteinase affecting gelatin, the four hemotoxins were tested for their ability to hydrolyze X-ray film emulsion. The effects of the hemotoxins on the X-ray film emulsion were estimated by chromatographing the hydroyzed emulsion (two dimensional chromatography) on silica gel H and spraying the plates with a 0.03 %

TABLE 18. Hemolysis range of isolated toxins.

Species RBC	Bands 2	3	4	5
Mouse	M	S*	S	M
Chicken	-	W	W	-
Guinea pig	-	M	W	VW
Cattle	-	S	S	VW
Sheep	-	W	W	VW
LV	A	T	T	A

M = Moderate
S* = Strong
W = Weak
VW = Very weak
- = No reaction
A = Atypical reaction (no fat)
T = Typical LV reaction (with fat)

TABLE 19. Hemolysis range of isolated toxins; "Hot-cold" effects.

Dilution	Band	Mouse		Cattle		Sheep**		Guinea pig	
		H*	H&C*	H	H&C	H	H&C	H	H&C
Undiluted	2	-	80	-	-	-	-	-	-
	3	+	+	20	90	-	90	-	t
	4	90	-	-	20	-	80	-	HA
	5	70	70	-	t	-	70	-	-
	Control	-	-	-	-	-	-	-	-
1:2	2	-	20	-	-	-	-	-	-
	3	+	+	t	+	-	+	-	t
	4	+	+	-	80	-	+	-	-
	5	50	80	-	20	-	30	-	-
	Control	-	-	-	-	-	-	-	-
1:4	2	-	t	-	-	-	-	-	-
	3	+	+	-	90	-	+	-	t
	4	70	90	-	80	-	80	-	-
	5	50	50	-	t	-	t	-	-
	Control	-	-	-	-	-	-	-	-

H* = Reaction at 30 C
H&C = Hot-cold reaction
Numbers = Percentage estimate of hemolysis
t = Trace of hemolysis
HA = Hemagglutination
\+ = Complete hemolysis
\- = No hemolysis

** For these cells had to use saline without borate because they hemolyse with the buffer.

alcholoic solution of ninhydrin. It was observed that the band which reacted with the gelatin and digested stromates of erythrocytes liberated two ninhydrin positive substances from the film emulsion. These substances were not released by a solution of band #2 which was heated at 95 C for 15 minutes or by the other hemotoxins. A drawing of the chromatoplate is shown in Figure 6.

Some studies on the isolated proteinase. Because the band containing the proteinase was generally contaminated with some electrophoretically slow-moving materials it was felt that the isolated diffusates should be tested for homogenity of reacting substances. Portions of the diffusates were tested for effects caused by trypsin inhibitor (Sigma Chemical Co., Type II-O) and ethylene diamino tetraacetic acid. The results of this experiment are shown in Table 20 where it can be seen that the EDTA inhibited hemolysis but not hydrolysis of X-ray film emulsion. Trypsin inhibitor, on the other hand, prevented hydrolysis of X-ray film emulsion but did not prevent hemolysis. All controls for the experiment were satisfactory. From this experiment it can be seen that the isolated proteinase may not be hemolytic. The significance of these findings is discussed in a later section of this thesis.

Identification of a lipase. Band #5, the fastest moving of the electrophoretically isolated hemolysins, reacted with butter oil to liberate a small amount of acid. The other hemotoxins did not react in a similar manner. The observation suggested that band #5 was a lipase. Some of the isolated hemotoxins were tested for lipase activity as described in page 26. The results are shown in Table 21 where it can be seen that the

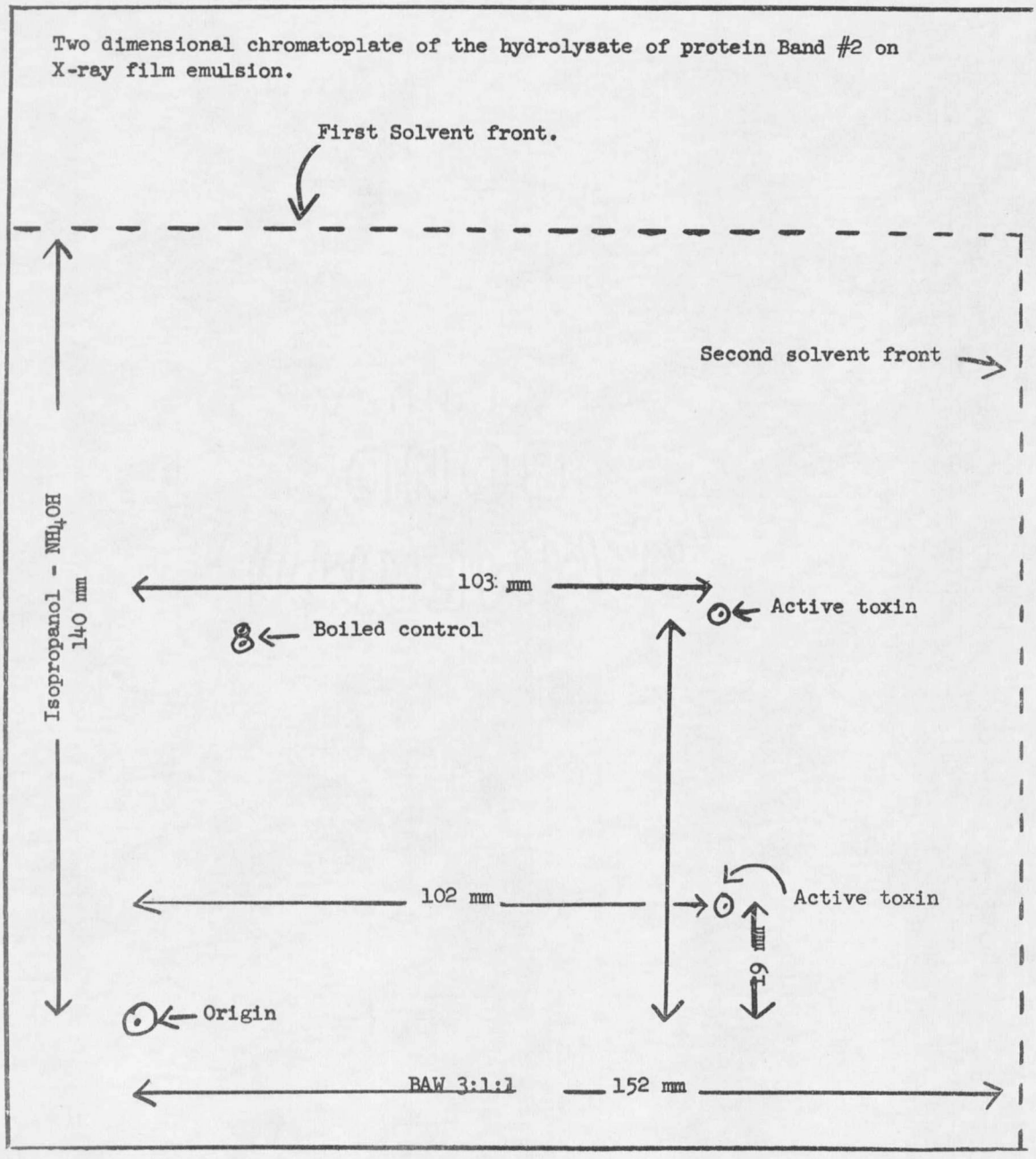


FIG. 6. Proteinase test of C. hemolyticum 7170 band 2.

TABLE 20. Experiments with proteinase (Band #2); effects of EDTA and trypsin inhibitor on X-ray emulsion.

Sample	Proteinase activity
Band #2 + Ca++	Positive reaction
Band #2 + Ca++ + EDTA	Positive reaction
Band #2 Trypsin inhibitor*	Negative reaction
Band #2 Boiled 15 minutes	Negative reaction
Gel + Trypsin inhibitor	Negative reaction
Calcium chloride soln.	Negative reaction

Effects of Ca++ and trypsin inhibitor on hemolysis.

Sample	Hemolytic activity
Band #2 + Ca++	Positive
Band #2 alone	Positive
Band #2 + Ca++ and EDTA	Negative
Band #2 and Trypsin inhibitor + Ca++	Positive
Trypsin inhibitor alone	Negative
Gel plus Ca++	Negative

* Sigma Chemical Co., Type II-O.

TABLE 21. Lipase activity of partially purified toxins of C. hemolyticum.

Substrates	Samples tested				
	4I	4 Act.	5I	5 Act.	Gel Dif.
None	-	-	-	-	-
Butter oil	-	-	-	?	-
Tween 20	-	-	-	-	-
Tween 65	-	-	-	-	-
Tween 85	-	-	-	+	-
Yolk	-	*	-	+*	-
Cream	-	-	-	-	-

* = Ring of whitish precipitate around well that does not react with copper sulfate.

+* = Ring of precipitate that reacts with the copper salt.

Tween 20 = Polyoxythylene sorbitan monolaureate
Tween 65 = Polyoxythylene sorbitan tristearate
Tween 85 = Polyoxythylene sorbitan trioleate

fastest moving hemolysin reacted with sorbitan trioleate (Tween 85) and with egg yolk.

Necrotizing and lethality potential of the hemotoxins. In this study the partially purified toxins were inoculated into the shaved abdomens of mice. The intracutaneous inoculations were given in 0.05 ml amounts. It was found that only the most reactive lecithinase C was necrotic, while the other substances had no effect on the inoculated animals. The results are shown in Table 22.

For the lethal studies, 0.3 ml amounts of substances diffused from each of the bands was inoculated intravenously into 20 g mice, but none of the animals died, although animals inoculated with the active lecithinase C, hemotoxin isolated from band #3, sickened within 3 hours after inoculation (See Table 22). The mice were sacrificed 18 hours after the inoculations and the following observations were made: Musculature over the thorax and the diaphragm were discolored, and adjacent to the sternum there was a small amount of orange-red edema. During the early hours of sickness the animals had ruffled coats and breathed fast; they moved very little and neither drank nor ate.

TABLE 22. Effect of intracutanous and intravenous inoculations of isolated toxins.

Band #	Route of inoculation	Amount of inoculation	Effect
2	Intracutaneous	0.05 ml	None
3	"	"	2/2*
4	"	"	None
5	"	"	None
2	Intravenous	0.3 ml	None/2
3	"	"	2 Sick/2
4	"	"	None
5	"	"	None

Diluent and gel diffusate controls: No effect.

* Red skin at end of 24 hours
Grayish color at 48 hours
Scab of yellowish-gray begins to form at 72 hours

DISCUSSION

The preliminary investigations for the present work were undertaken to study the hypothesis that organisms producing lecithinase C should produce culture fluids that yield proportional hemolytic and lethal reactions. The task was initiated because some of the data in the literature were contradictory and the author felt that the results were not in agreement because the hemolysis reactions were carried out with erythrocytes of species other than those used for the lethality assays. The contradictions were particularly noticeable in work with culture fluids of C. bifermentans, (Miles and Miles, 1947; 1950) C. perfringens type A, (Dolby and macfarlane, 1956) and B. cereus (Chu, 1949).

The initial experimental work was conducted with culture fluids of C. hemolyticum, C. perfringens type A, C. sordellii and B. cereus. With these toxic fluids it was learned that there was no correlation between lethality and hemolysis when mice and chickens and their erythrocytes were used. Subsequent work was limited to investigations with culture fluids of C. hemolyticum which theoretically should have produced but one substance causing hemolysis, LV or lethality. Work with C. perfringens type A was discontinued because it was known that this organism produces a number of toxic substances which could have reacted with egg yolk or erythrocytes; work with C. sordellii was discontinued because the organism was a very poor producer of lecithinase C; and work with B. cereus was discontinued because the organism produced a hemolysin whose activity was not dependent on the presence of calcium.

With a number of culture fluids of C. hemolyticum it was verified

that new-born chickens and 20 g mice were about equally susceptible to the effects of toxic fluids. It was also verified that the chicken erythrocytes were much more resistant to hemolysis than those of mice.

The LV reaction end-points for the present studies were observed to be in closer agreement with lethality estimates than were the hemolysis end-points. Because proteinases, lipases, phospholipases and chemicals can cause LV type reactions (Macfarlane and Knight, 1941; Crook, 1942; Willis, 1960; van Heyningen, 1941) and since C. hemolyticum was found to produce all the above enzymes, it may seem strange that LV should bear a relationship to lethality. The unexpected relationship may be explained by the fact that C. hemolyticum was found to produce predominantly phospholipase C (Band #3) which readily reacted with yolk and which was the main attribute of the toxicity of the culture fluids.

If the hypothesis that hemolysis is proportional to lethality had been investigated using mammals and their respective erythrocytes, it is possible that this relationship would have been established. In this study, chicken erythrocytes were used and though they contain lecithin and sphingomyelin (Turner, 1957), the cells were very resistant to hemolysis and the sought relationship was not obtained. It is the author's opinion that the resistance could possibly be due to one or more of the following conditions: (1) the tight binding of phospholipids with proteins in the stroma (Turner, 1957) which would make the substrate inaccesible to the hemolysins, (2) the presence of phytic acid in chicken cells (Rapoport, 1940) which would complex with calcium and thus interfere with the reaction of the hemolysins, and (3) the relatively small amounts of fats in the

structures (Erickson, 1938) which may be necessary for the hemolysins to react with proper substrates in the stroma of the erythrocytes.

While the chemical composition of erythrocytes of various animals and man has been studied more or less extensively (deGier and van Deenen, 1961b; Turner et al., 1958; Behrendt, 1957; Ponder, 1948; Plankerd, 1961), similar studies with mouse erythrocytes have not been undertaken. Any attempt to compare the chemical reactions of hemolysis of mouse erythrocytes with those of chicken would be strictly speculative, but since mouse erythrocytes are so much more sensitive to hemolysis by a number of toxins, it is tempting to postulate that the fragility of these cells is due to loose binding between the lipids and proteins of the stroma.

An assumption that hemolysis should be proportional to lethality may not necessarily be appropriate. First, it is known that the phospholipid composition of erythrocytes need not be representative of the chemical composition of all the cells in the body (Matsumoto, 1961a). Second, it is known that a lecithinase C affects the erythrocytes of more species than substrates in sera of the same species (Nagler, 1939; Oakley and Warrack, 1941; Dolby and Macfarlane, 1956). Third, it is known that many of the erythrocytes exhibit the "hot-cold" phenomenon on treatment with fluids of C. hemolyticum (Smith, 1954) and this reaction could alter the ratio of hemolysis to lethality.

With a culture grown in a cellophane sack it was seen that the hemolysis reaction with mouse cells was linear with varying concentrations of the toxic preparation. The hemolysis pattern with chicken erythrocytes was irregular and was characterized by an initial slope of hemolysis

followed by a flat plateau and then by a second slope of hemolysis. The observation with the chicken erythrocytes suggested that the toxic preparation contained at least two hemolysins. The irregular hemolysis pattern with chicken erythrocytes was verified with a number of other preparations. No correlation between hemolysis and lethality could be established.

A preparation of toxic culture fluid, identified as Batch No. 5, was fractioned electrophoretically by a modification of the Ornstein and Davis technique (1960). It was seen that the resultant gel columns contained about seven bands of protein that were not seen in medium control preparations. Five of the seven bands were hemolytic, but work was limited to identification of four of these bands. The fifth hemolytic band was not studied because it did not contain sufficient extractable toxin. The remaining hemolytic substances were subjected to further study and it was found that two of the bands were phospholipases. The hemolytic band exhibiting the greatest mobility in the acrylamide gel was a lipase that hydrolyzed synthetic sorbitan trioleate, and the slowest moving hemolysin (Band #2) was a mixture of at least two proteinaceous substances. Further separation of these materials was impossible with the techniques used, but the hemolysin was serologically related to the phospholipases and was not necrotizing or lethal. The slow moving mixture also contained a proteinase that hydrolyzed gelatin and the emulsion of X-ray film. From hydrolyzed emulsion it was possible to see, by means of chromatography, that two substances were removed from the emulsion, an amino acid with an RF value similar to that of isoleucine and a peptide.

From the foregoing observation the lipase postulated by Macfarlane (1950b) was identified as an enzyme that hydrolyzed lipids containing oleic acid. It was also seen that a number of hemolysins were isolated and that their presence may well have contributed to the irregular hemolytic reactions (Figures 4 and 5). Since the isolated hemolysins had different host ranges for various erythrocytes, we are left with M. G. Macfarlane's "goodness of fit" concept (1950b). No effort was made to confirm the hypothesis of Bard and McClung (1948) that C. hemolyticum produces a lysolecithin because the main objective of the latter part of this study was to find an answer to the lack of correlation between hemolysis and lethality.

It was observed that all the hemolytic reactions required calcium. This suggests that all the preparations tested could have been contaminated with the same hemolysin, with the possible exception of the isolated lipase which was serologically different from the other hemotoxins. Because great care was taken to assure the purity of the materials tested, it is this author's opinion that if the isolated substances were impure, they may be "natural mixtures" where a single hemolysin can combine with a number of proteins including some of the isolated toxins. The multiplicity of combinations could result in a number of similar hemolysins with different electrophoretic mobilities.

A curious thing about many hemolytic reactions is that they require calcium (Mayer and Levine, 1954), and since this phenomenon is also observed in immune hemolysis, it is tempting to believe that any one of a variety of calcium dependent reactions can alter the integrity of the

stromata. The alteration can serve to trigger dormant enzymes present in the structures with a resultant increase in hemolysis. With this concept in mind it can be visualized that Bard and McClung's (1948) hypothesis that C. hemolyticum contains a lysolecithin may be incorrect to the extent that the lysolecithin may have been derived from erythrocytes. The stroma of erythrocytes are known to contain lysophosphatides (deGier and van Deenen, 1961b).

Further work with chromatography and electrophoresis would be of value in reducing the possible experimental errors presented in this thesis and would aid in advancing knowledge about the nature of bacterial toxins.

SUMMARY

Work with culture fluids of Clostridium hemoliticum was divided into two main categories; (a) Studies on crude toxin perparations, and (b) Studies on electrophoretically purified toxins.

Studies with the crude toxin fluids of C. hemolyticum were designed to test a hypothesis that there would be a proportional relationship between lethality of an inoculated species and in vitro hemolysis of erythrocytes of the same species. The hypothesis was tested with chickens and mice which are well separated phylogenetically. The animals were inoculated intravenously and intramuscularly and the 50% lethal end-points were calculated by the method of probits. Erythrocytes of these species were mixed with varying concentrations of the same toxin used for the lethality assays. The 50% hemolytic end-points were estimated from multiple titrations.

It was observed that adult mice and new-born chickens were about equally susceptible to the effects of the toxins but that the erythrocytes of mice were much more susceptible to hemolysis than were the erythrocytes of chickens. Since the amount of hemolysis could be affected by cellular fragility it was decided to control this condition by measuring the amount of acid soluble phosphate liberated from exposed erythrocytes. It was found that this approach was unsatisfactory because no phosphate was detected from hemolyzed chicken cells.

Lecithovitellin reactions of these toxin preparations were conducted to study some of the conditions necessary for optimal reaction of the fluids, and to compare these reactions with hemolysis and toxicity.

A number of toxin fluids were prepared to see if all would react similarly with egg yolk and the erythrocytes of mice and chicken. These toxin substances yielded linear hemolysis patterns with mouse cells, but the patterns with chicken cells were all similar but irregular if they are compared to the "normal" sigmoid hemolysis curves. While conducting some of these tests it was seen that in some toxic preparations the LV/ hemolysis ratio was not proportional for all the toxins.

The electrophoretically treated toxin fluids showed that C. hemolyticum produced a number of hemolysins. Some of the substances were isolated and were identified as a proteinase, two phospholipases and a lipase. The proteinase was one of the slower moving proteins, hydrolyzed emulsions of X-ray film and could be inactivated by heat. The proteinase was not obtained in a pure state because of the very close proximity of other protein bands in the gel columns.

The lipase was a weak hemolysin which hydrolyzed synthetic sorbitan trioleate to release a small amount of titratable acid. This substance was serologically different from the other hemolysins isolated, and reacted with egg yolk suspensions to produce atypical LV reactions.

The slowest moving of the isolated phospholipases, a very active hemolysin, was necrotic, and in the small amounts available was almost lethal when given intravenously to mice. The faster moving phospholipase was serologically very closely related to the former. Both of these compounds reacted with egg yolk to produce typical LV type reactions.

Although the work with electrophoresis helped to explain some of the observed discrepancies between hemolysis and lethality, different cellular fragility between the mouse and chicken erythrocytes may also have been involved. Further work with electrophoresis and column chromatography would be of great value in verifying the present work and advancing knowledge of bacterial toxins.

LITERATURE CITED

Bard, R. C. and B. S. McClung. 1948. Biochemical properties of the toxins of Clostridium novyi and Clostridium hemolyticum. J. Bacteriol. 56: 665-670.

Bobbitt, J. M. 1963. Thin Layer Chromatography. New York. Reinhold, Pub. p 30-105.

Bullen, J. J. and G. H. Cushnie. 1963. The failure of antitoxin to protect guinea-pigs against intraperitoneal infection with Clostridium welchii type A. J. Pathol. Bacteriol. 86: 345-360.

Costlow, R. D. 1958. Lecithinase from Bacillus anthracis. J. Bacteriol. 76: 317-325.

Crook, E. M. 1942. The Nagler reaction: the breakdown of lipoprotein complexes by bacterial toxin. British J. Expt. Pathol. 23: 37-55.

Chu, J. 1949. The lecithinase of B. cereus and its comparison with Cl. welchii alpha toxin. J. Gen. Microbiol. 3: 255-273.

de Gier, J., G. H. de Haas and L. L. M. van Deenen. 1961a. Action of phospholipases from Clostridium welchii and Bacillus cereus on red cell membranes. Biochem. J. 81: 33p-34p.

de Gier, J. and L. L. M. van Deenen. 1961b. Some lipid characteristics of red cell membranes of various animal species. Biochim. Biophys. Acta. 49: 286-296.

de Haas, G. H. and L. L. M. van Deenen. 1961. Synthetic mixed-acid Kephalins; hydrolysis by phospholipase A and some properties of their sodium salts. Biochem. J. 81: 34p-35p.

Deverell, M. W. 1957. Studies on the metal requirements of Clostridium perfringens lecithinase using chelating agents. Ph.D. thesis. Purdue University.

Dolby, D. E. and M. G. Macfarlane. 1956. Variation in the toxicity of lecithinases (Alpha toxin) from different strains of Clostridium welchii. British J. Expt. Pathol. 37: 324-332.

Erickson, B. N., H. H. Williams, S. S. Bernstein, I. Avin, R. L. Jones, and I. G. Macy. 1938. The lipid distribution of posthemolytic residue or stroma of erythrocytes. J. Biol. Chem. 122: 515-528.

Evans, D. G. 1943. The protective properties of the alpha antitoxin and theta antihaemolysin occuring in Cl. welchii type A antiserum. British J. Expt. Pathol. 24: 81-88.

Evans, D. G. 1945. The in-vitro production of Alpha toxin, hemolysin and hyaluronidase by strains of Cl. welchii Type A, and the relationship of in-vitro properties to virulence for guinea-pigs. J. Pathol. Bacteriol. 57: 75-85.

Finney, D. J. 1952. Probit analysis. Cambridge University Press, London.

Fiske, C. H. and Y. Subbarow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375-400.

Fuzi, M. and I. Pillis. 1962. Production of opacity in egg-yolk medium by Listeria monocytogens. Nature (London) 196: 195.

Glenny, A. T., M. Barr, M. Llewellyn-Jones, T. Dalling, and H. E. Ross. 1933. Multiple toxins produced by some organisms of the Cl. welchii group. J. Pathol. Bacteriol. 37: 53-74.

Gillespie, W. A. and V. G. Alder. 1952. Production of opacity in egg yolk media by coagulase-positive staphylococci. J. Pathol. Bacteriol. 64: 187-200.

Hall, I. C. 1929. The occurrence of Bacillus sordellii in icterchemoglobinuria of cattle in Nevada. J. Infect. Diseases 45: 156-162.

Hayward, N. J. 1943. The rapid identification of Cl. welchii by Nagler tests in plate cultures. J. Pathol. Bacteriol. 55: 285-293.

Heimpel, A. M. 1955. Investigations of the mode of action of strains of Bacillus cereus Fr. and Fr. pathogenic for the larch sawfly, Pristiphora erichsonii. Can. J. Zool. 33: 311-326.

Hugo, W. B. and E. G. Beveridge. 1962. A quantitative and qualitative study lipolytic activity of single strains of seven bacterial species. J. Appl. Bacteriol. 25: 72-82.

Jasmin, A. M. 1947. Enzyme activity in Clostridium hemolyticum toxin. Am. J. Vet. Res. 8: 289-293.

Jones, F. M. and J. J. Gleiser. 1954. Veterinary necropsy procedures. J. B. Lippincott Co. p 63-81.

Kielley, W. W. and O. Meyerhof. 1950. Studies on adenosinetriphosphate of muscle III. The lipoprotein nature of the magnesium-activated adenosinetriphosphatase. J. Biol. Chem. 183: 391-401.

Kushner, D. J. 1957. An evaluation of the egg yolk reaction as a test for lecithinase activity. J. Bacteriol. 73: 297-302.

Kushner, D. J. and A. M. Heimpel. 1957. Lecithinase production by strains of Bacillus cereus Fr. and Fr. pathogenic for the larch sawfly, Pristiphora erychsonii (HTG). Can. J. Microbiol. 3: 547-511.

Lazear, E. J., A. H. Killinger, M. B. Hays, and H. Engelbrecht. 1958. The Gel precipitin test 1. Its technique and applications in veterinary medicine. Veterinary Med. 53: 229-235.

Levine, L., K. M. Cowan, A. G. Osler, and M. M. Mayer. 1953. Studies of the role of Ca++ and Mg++ in complement fixation and immune hemolysis. III the respective roles of Ca++ and Mg++ in immune hemolysis. J. Immunol. 21: 374-379.

Lowry, O. H., A. L. Rosebrough, A.L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Macfarlane, M. G. and B.C.J.G. Knight. 1951. The biochemistry of bacterial toxins. I. The lecithinase activity of Cl. welchii toxins. Biochem. J. 35: 884-902.

Macfarlane, M. G. 1942. The lecithinase activity of Cl. oedematiens toxins. Biochem. J. 36: iii.

Macfarlane, M.G. 1950a. The biochemistry of bacterial toxins. 4. The lecithinase activity of Clostridium haemolyticum toxin. Biochem. J. 47: 267-270.

Macfarlane, M. G. 1950b. The biochemistry of bacterial toxins. 5. Variation in haemolytic activity of immunologically distinct lecithinases towards erythrocytes from different species. Biochem. J. 47: 270-279.

MacFarlane, R. G., C. L. Oakley, and C. G. Anderson. 1941. Haemolysis and the production of opalescence in serum and lecithovitellin by the alpha toxin of Clostridium welchii. J. Pathol. Bacteriol. 52:99-103.

Mack, W. B. and W. Records. 1917. Studies of an obscure cattle disease in Western Nevada. J. Am. Vet. Med. Assoc. 3: 143-55.

Martland, M. and R. Robinson. 1926. Possible significance of hexose phosphoric esters in ossification. IV. Phosphoric esters in blood-plasma. Biochem. J. 20: 847-55.

Matsumoto, M. 1961a. Studies on phospholipids. I. Comparative analysis of phospholipids from mammalian blood stroma and spleen. J. Biochem. (Tokyo) 49: 11-22.

Matsumoto, M. 1961b. Studies on phospholipids. II. Phospholipase activity of Clostridium perfringens toxin. J. Biochem. (Tokyo) 49: 23-31.

Mayer, M. M. and L. Levine. 1954. Kinetic studies on immune hemolysis. IV. Rate determination of the Mg++ and terminal reaction steps. J. Immunol. 72: 516-530.

McClung, L. S. and R. Toabe. 1947. The egg-yolk plate reaction for the presumptive diagnosis of Cl. sporogenes and certain of the gangrene and botulimum groups. J. Bacteriol. 53: 139-47.

Meyer, K. F. 1916. Studies to diagnose a fatal disease of cattle in the mountainous regions of California. J. Am. Vet. Med. Assoc. (NS) 1: 552-560.

Miles, E. M. and A. A. Miles. 1947. The lecithinase of Clostridium bifermentans and its relation to the toxin of Clostridium welchii. J. Gen. Microbiol. 1: 385-399.

Miles, E. M. and A. A. Miles. 1950. The relation of toxicity and enzyme activity in the lecithinases of Clostridium bifermentans and Clostridium welchii. J. Gen. Microbiol. 4: 22-35.

Nagler, F. P. O. 1939. Observations on the reaction between the lethal toxin of Clostridium welchii (type A) and human serum. British J. Expt. Pathol. 20: 473-485.

Oakley, C. L. and G. H. Warrack. 1941. Factors affecting the activity of the alpha toxin of Clostridium welchii. J. Pathol. Bacteriol. 53: 335-370.

Oakley, C. L. 1943. The toxins of Clostridium welchii. A critical review. Bull. Hyg. 18: 781-806.

Oakley, C. L., G. H. Warrack, and P. H. Clarke. 1947. The toxins of Clostridium oedematiens (Cl. novyi). J. Gen. Microbiol. 1: 91-106.

Oakley, C. L. and H. G. Warrack. 1959. The soluble antigens of Clostridium oedematiens type D (Cl. haemolyticum). J. Pathol. Bacteriol. 78: 543-551.

Ornstein, L. and B. J. Davis. 1960. Disc electrophoresis. Preprinted by Distillation Product Industries Div. Eastman Kodak. Co., Rochester, N. Y.

Pangborn, M. C. 1950. A simplified purification of lecithin. J. Biol. Chem. 188: 71-76.

Patel, V., H. S. Goldberg, and D. C. Blenden. 1963. Characterization of leptospiral lipase (esterase). Bacteriol. Proc. 63rd An. Meeting. P. 105.

Ponder, E. 1948. Hemolysis and related phenomena, New York, Grune and Stratton.

Prankerd, T. A. J. 1961. The red cell. Springfield, Ill. Charles C. Thomas, Pub. p 162-168.

Rapoport, S. 1940. Phytic acid in avian erythrocytes. J. Biol. Chem. 135: 403-406.

Records, E. and L. R. Vawter. 1921. Ictero-hemoglobinuria in cattle. J. Am. Vet. Med. Assoc. 13: 155-164.

Records, E. and L. R. Vawter. 1945. Bacillary hemoglobinuria of cattle and sheep. Univ. Nev. Agr. Exp. Sta. Bull. 173.

Rogols, S., W. B. Fizette, and E. H. Bohl. 1959. Studies on the relationship of a phospholipase to the hemolysins of Leptospira pomona. J. Vet. Res. 20: 592-596.

Ruata, G. Q. and G. Caneva. 1901. Della scomposizione delle lecitine. Ann. dIgiene 11: 341-354.

Sebald, M. and A. R. Prevot. 1960. Etude qualitative et quantitative du pouvoir lipidolitique de 86 souches de Clostridium anaerobies. Ann. Inst. Pasteur 99: 386-400.

Shah, D. B. and J. B. Wilson. 1962. Egg yolk factor of Staphylococcus aureus I. Nature of the substrate and enzyme involved in the egg yolk opacity reaction. J. Bacteriol. 85: 516-521.

Smith, L. DS. and M. V. Gardner. 1950a. The inhibition of the lecithinase of Clostridium perfringens by some reducing agents. J. Franklin Inst. 250: 465-71

Smith, L. DS. and M. V. Gardner. 1950b. The anomalous heat inactivation of Clostridium perfringens lecithinase. Arch. Biochem. 25: 54-60.

Smith, L. DS. 1953. The essential characteristics of the species Clostridium hemolyticum. J. Bacteriol. 65: 222.

Smith, L. DS. 1954. Introduction to the pathogenic anaerobes. The Univ. of Chicago Press.

Turner, J. C. 1957. Absence of lecithin from the stromata of the red cells of certain animals (ruminants) and its relation to venom hemolysis. J. Exp. Med. 105: 189-193.

Turner, J. C., H. M. Anderson, and C. P. Gandal. 1958. Specied differences in red cell phosphatides separate by paper and column chromatography. Biochim. Biophys. Acta. 30: 130-134.

Vawter, L. R. and E. Records. 1926. Recent studies on ictero-hemoglobinuria of cattle. J. Am. Vet. Med. Assoc. 21: 494-512.

Vawter, L. R. and E. Records. 1929. Immunization of cattle against Bacillary hemoglobinuria. J. Am. Vet. Med. Assoc. 28: 201-214.

Vawter, L. R. and E. Records. 1931. Serologic study of sixteen strains of Bacillus hemolyticus. J. Infect. Diseases 48: 581-587.

van Heyningen, W. E. 1941. The biochemistry of the gas gangrene toxins. I. Estimation of the alpha toxin of Cl. welchii type A. Biochem. J. 35: 1246-1256.

Willis, A. T. 1960. The lipolytic activity of some clostridia. J. Pathol. Bacteriol. 80: 379-390.

Willis, A. T. and G. Gowland. 1962. Some observations on the mechanism of the Nagler reaction. J. Pathol. Bacteriol. 83: 219-226.

Willis, A. T. and G. C. Turner. 1962. Staphylococcal lipolysis and pigmentation. J. Pathol. Bacteriol. 84: 337-347.

Woolridge, W. R. and C. Higginbottom. 1938. The effect of certain bacterial toxins upon some respiratory mechanisms of animal tissues. Biochem J. 32: 1718-1728.

MONTANA STATE UNIVERSITY LIBRARIES
3 1762 10010890 9

D378
L959
cop. 2
Lozano, Edgardo A
The hemolysins of
Clostridium hemolyticum

NAME AND ADDRESS

Frank Carr
418 W. Garfield

1-12-69 Ted Odle Merriam
Lab use 10128 W 65th KS

D378
L959
cop. 2

BOUND BY HNM