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
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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.

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Head, Major Department

Chairman, Examining Committee

Dean, Graduate Division

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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.

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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