THESIS

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THE ISOLATION AND PARTIAL CHARACTERIZATION OF AN ENTEROTOXIN PRODUCED BY *AEROMONAS HYDROPHILA* AND *AEROMONAS SOBRIA*

By

Dace Valduss

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ABSTRACT

THE ISOLATION AND PARTIAL CHARACTERIZATION OF AN ENTEROTOXIN PRODUCED BY *AEROMONAS HYDROPHILA* AND *AEROMONAS SOBRIA*

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The suckling mouse assay was used to test for enterotoxic activity with human and environmental isolates of *Aeromonas hydrophila* and *Aeromonas sobria*. Enterotoxic activity could be detected in 5 to 9 isolates. Three of these isolates were *Aeromonas sobria* and 2 were *Aeromonas hydrophila*. Negative strains remained negative for enterotoxic activity even after ten-fold concentration of the supernatant. Cell free filtrates tested in suckling mice produced a positive response indicating that the enterotoxin is a cell-free exotoxin released into the growth media. When heated at 60°C for 20 minutes, the filtrates lost enterotoxic potential suggesting that the toxin is heat labile. Column chromatography performed with concentrated enterotoxin indicated that the toxin is a large molecular weight substance, perhaps protein in nature. Source of isolation appears not to play a role in enterotoxin production since both enrivonmental and human isolates were reactive in the assay.

DEDICATION

To my parents

Visvaldis and Ella Valduss

and my grandmother

Alma Rumba

Thank you for your encouragement, love, and understanding.

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INTRODUCTION

In recent years, organisms previously considered saprophytic or non-pathogenic for man have been implicated as the cause of human disease. Many investigators call attention to the increasing numbers of infections caused by Aeromonas hydrophila (20, 52, 60, 75, 85). The majority of bacteremic infections have occurred in patients with chronic hepatic disease or with an underlying neoplastic or hematologic disorder (60, 85, 88). Epidemiologically, Aeromonas has been isolated primarily from non-fecally contaminated fresh water, city tap water and rural rivers, lakes and canals (3, 4, 45). Aeromonads seem to thrive in water characterized by a high content of non-fecal organic waste material. They have also been isolated from fecal specimens from children and adults with diarrhea of unexplained origin as well as from healthy carriers (3, 4, 24).

The primary objective of this study was to examine clinical and environmental isolates of Aeromonas hydrophila and Aeromonas sobria for enterotoxic activity in the suckling mouse assay. After isolating a suckling mouse reactive "toxin", further studies were performed to define the nature of this enterotoxic material with regard to filterability, ability to be retained on ultrafiltration (PM30) membranes and stability to heat. Attempts were also made to correlate other parameters such as cytotoxic activity, piliation, motility and LD_{50} with enterotoxic activity.

LITERATURE REVIEW

A Brief History of Aeromonas

Bergey's Manual defines the genus Aeromonas as follows: "Short (rarely more than 3 microns), rod shaped cells. Motile by means of polar flagella, usually monotrichous: occasionally non-motile. Gram negative, carbohydrates fermented with the production of H_2, O_2 and 2,3 butylene glycol. Methyl red negative. Slow or no fermentation of lactose. The majority of the species thus far described are from water or are known to be pathogenic to marine and fresh water animals such as fish and amphibians." The manual lists four species: Aeromonas liquefaciens, Aeromonas puncatata, Aeromonas hydrophila and Aeromonas salmonicida.

Strains of bacteria which are now considered to belong to the genus Aeromonas have been isolated and described in some detail during the past 70 years (26, 27, 71, 83). Many have undergone numerous name changes as well as changes in grouping. Since much of the early literature is confusing, a short description of each of the 4 main species of Aeromonas is provided (based on Eddy's 1960 review of the literature and history of Aeromonas [26]).

Aerobacter liquefaciens (Aeromonas liquefaciens) was first isolated by Beijerinck in 1900 from the ditches of the Delft. He proposed the generic name Aerobacter and described the organism as having a single polar flagellum and the ability to rapidly liquefy gelatin. In 1936

Kluyver and van Niel proposed that the name be changed to Aeromonas. This species was included, for the first time, in the 7th edition of Bergey's Manual as Aeromonas liquefaciens.

Bacillus punctatus (Aeromonas punctata) was first isolated from tap water by Zimmerman in 1890. In the first five editions of Bergey's Manual, it was known as Achromobacter punctatum. In the 6th edition it became Pseudomonas punctata and in the 7th edition Aeromonas punctata. Some strains are very pathogenic to fish while others are nonpathogenic.

Bacterium salmonicida (Aeromonas salmonicida) was first isolated by Emmerich and Weibel in 1894. It is non-motile and produces a pigment. This organism differs from other Aeromonads by being Voges-Proskauer negative and psycrophilic.

Bacillus hydrophilus fuscus (Aeromonas hydrophila). The earliest strains were isolated from diseased frogs. The first person to investigate septicemic diseases of frogs was Ernst, who in 1890 reported the isolation of a bacterium from the blood of infected frogs. He named the organism Bacillus ranicida. A year later, Sanarelli described an organism which he isolated from the lymph and blood of frogs. He isolated the same organism from the laboratory water supply. Sanarelli named his organism Bacillus hydrophilus fuscus, since he did not believe it to be identical to Bacillus ranicida. Sanarelli's bacteria was pathogenic for frogs, toads, salamanders, lizards, sunfish and fresh water eels. Injection of this organism into warm blooded animals such as guinea pigs, rabbits, dogs, cats, mice, chickens and pigeons resulted in death within a relatively short time. In 1893, Trambusti isolated the same species from diseased frogs in his laboratory. His investigations were primarily

concerned with the isolation of toxic metabolic products from the organism. His experiments indicated that the toxic products were of two kinds, one soluble and the other insoluble in alcohol. He attempted to determine the physiologic action of pure cultures and toxins of the organisms on experimental animals. Roger in 1893 described an epizootic disease among frogs characterized by a distinct reddening of the legs. He found that the viscera and blood of the frogs contained a small bacillus in pure culture. The same organism was isolated from the water of the aquarium which housed the infected frogs. Roger concluded that the organism was the same organism isolated by Sanarelli. In the 1st edition of Bergey's Manual the organism was named Proteus hydrophilus and described as having peritrichous flagella. Russel had previously shown that the organism had a single polar flagellum. This error remained until the 6th edition when the organism was placed in the genus Pseudomonas. In 1943, Stanier proposed that the genus be changed to Aeromonas. The first description of the morphological, cultural, biochemical and pathogenic properties of Aeromonas hydrophila were described by Russel in 1898. He confirmed much of the findings of Sanarelli and Trambusti. He found the organism produced two potent toxins, one of these resembling digitalis in its action and the other veratrin. Eddy in 1960 suggested revisions for the genus Aeromonas. He proposed combining the species Aeromonas liquefaciens, Aeromonas hydrophila and Aeromonas punctata into a single group, Aeromonas liquefaciens. Those organisms which are polarly flagellated, methyl red positive, Voges-Proskauer negative and anaerogenic would be called Aeromonas formicans. He also recommended that the name Aeromonas salmonicida be retained for the

non-motile, Voges-Proskauer negative and psycrophilic Aeromonads. Ewing, Hugh and Johnson proposed a reorganization of the genus Aeromonas that differed from Eddy (61). They proposed that the genus contain three species: Aeromonas salmonicida, Aeromonas shigelloides, and Aeromonas hydrophila (consisting of the genera Aeromonas liquefaciens and Aeromonas hydrophila). In 1962, after additional study, Eddy proposed further revisions (27). He would include the species Aeromonas liquefaciens, Aeromonas hydrophila, which he considered synonymous with Aeromonas punctata, under the heading Aeromonas punctata. Aeromonas formicans should be renamed Aeromonas caviae. Aeromonas salmonicida should be retained.

Aeromonas sobria was first described by Popoff and Veron (71). These organisms possess all the fundamental properties of the Aeromonads. They differ from Aeromonas hydrophila in that they do not utilize L-histidine, L-arabinose, L-arginine and salicin as a sole carbon source. They do not grow on KCN medium, hydrolyze esculin or ferment salicin. These authors also proposed the retention of Aeromonas hydrophila as a species. They believed that Aeromonas hydrophila could be divided into two subspecies: anaerogenes and hydrophila, based on glucose fermentation and butanediol dehydrogenase production. Boulanger, Lallier and Cousineau isolated Aeromonas sobria from healthy fish and confirmed the biochemical differences described by Popoff and Veron (13).

Aeromonas Infection in Frogs

Emerson and Norris first described the infection of frogs with Aeromonas as "red-leg disease" for the hemorrhagic lesions found on the

abdomen and legs of diseased frogs. Infection can result from spontaneous or artificial infection of frogs with Aeromonas hydrophila (55). The disease begins with a distinct sluggishness of the animal. Within a few days, hemorrhagic areas are observed on the ventral surface of the body. Extensive edema of the abdomen and thighs occurs several hours before the frogs succumb to the disease. Post mortem examination reveals edematous fluid beneath the skin of the abdomen and thighs. Small amounts of blood tinged exudate can be found in the mouth along with small hemorrhagic areas on the surface of the tongue. Multiple petechial hemorrhages are always seen on the surface of the thigh and abdominal musculature. The peritoneal cavity of infected frogs contains hemolyzed bloody exudate in which can be found large numbers of infecting organisms. The heart muscle appears pale and flaccid. The blood vessels on the surface of the stomach and intestines appear congested, while the organs appear distended. The liver is dark brown and mottled. The spleen becomes enlarged while the kidneys show no abnormal changes. Aeromonas hydrophila can be readily recovered from the organs, exudate and blood of infected frogs. The portal of entry seems to be abraided skin.

Aeromonas also causes black rot in hen eggs (9). It is pathogenic to a wide variety of fish (13). Aeromonas hydrophila has been isolated from septicemias in snakes and salamanders (9, 19). In the case of snake septicemias, the organism is transmitted from snake to snake by snake mites (19).

Aeromonas Infection in Humans

In humans, Aeromonas has been associated with gastro-enteriris (6, 14, 19, 43, 57, 75) cellulitis (88), septicemia (1, 28, 52, 65, 73, 74, 85),

endocarditis (20), meningitis (72), wounds (45, 60, 90), and urinary tract infections (39, 40). Aeromonads have also been isolated from the stools of healthy carriers (3, 4, 24). Patients presenting with gastrointestinal disturbances caused by Aeromonas hydrophila usually have a mild self limited diarrhea. Nausea, vomiting and abdominal pain have also been observed (88). Those patients who present with septicemia resemble septicemias caused by other gram negative organisms. These patients usually have an underlying hematologic disorder, are immunosuppressed or have liver disease (1, 14, 28, 58, 65, 74, 79, 85, 87). Septicemias caused by gram negative organisms in Laennec's cirrhosis are due to blood invasion of intestinal bacteria that have crossed a damaged intestinal mucosa, have bypassed the liver or have been insufficiently filtered from the portal blood by the liver (88). It appears that the gastrointestinal disturbances caused by Aeromonads may be due to an enterotoxin or cytotoxin produced by the bacteria (14, 43, 57, 78).

Enterotoxins of Gram Negative Bacteria

Vibrio cholera Enterotoxin

In 1884, Koch first suggested that cholera was a toxinosis. It is now firmly established as the prototype of the enterotoxic enteropathies. Toxin production in V. cholera is coded for by a chromosomal gene (87). The enterotoxin molecule has a molecular weight of 84,000 daltons and is composed of 2 subunits, A and B (34). Subunit A has a molecular weight of about 28,000 daltons and is responsible for the toxic activity of choleragen (cholera toxin). Subunit A can be further separated into two fragments: A_1 with a molecular weight of 23,000 daltons and A_2 with a

molecular weight of 2,500 daltons (67). Subunit B is the portion of the protein which binds to Gm_1 ganglioside in the outer membrane of intestinal cells and is not toxigenic (33). The proposed structure of cholera toxin is AB_5 or AB_6. Cholera toxin spontaneously disassociates into choleragenoid, an aggregation of B subunits, with a molecular weight of 68,000 daltons. It is immunogenic but not toxigenic. Cholera toxin activates membrane bound adenylate cyclase, which converts adenosine 5' triphosphate (ATP) to cyclic AMP. Elevated levels of cyclic AMP result in a net secretion of cholride and inhibition of sodium absorption resulting in loss of water. Cholera toxin is heat labile. Activity can be measured in suckling mice, rabbit ileal loops, skin permeability and Y_1 adrenal cells (7, 8, 12, 34, 69).

Escherichia coli Enterotoxins

Escherichia coli that produce diarrhea were formerly divided into three groups: enteropathogenic, enterotoxigenic and enteroinvasive. Enteropathogenic E. coli were identified by their somatic (O) and flagellar (H) antigens (46). During the 1940's and 1950's certain strains of E. coli identified by serotype were isolated from the stools of infants with diarrhea during outbreaks in hospital nurseries (44). These 14 distinct antigenic types were designated as the classical enteropathogenic serotypes. Since the 1950's, however, it was demonstrated that these classical serotypes were found in stool specimens from healthy infants as often as in stools from infants with diarrhea (51). Today there is considerable doubt about the importance of the enteropathogenic E. coli. Therefore, enteropathogenic E. coli are no longer considered to be a

separate group. The current literature distinguishes E. coli into two groups--enterotoxigenic and enteroinvasive.

Enterotoxigenic E. coli produce disease by elaboration of an exotoxin. Some strains of E. coli produce labile toxin (LT), stable toxin (ST) or both (44, 56, 82, 91). Toxin production is plasmid mediated. Stable toxin has been shown to have a molecular weight of approximately 4,400 (2, 84). Stable toxin contains a large number of cystine residues and has few hydrophobic amino acids (2). It is stable to heating at 100° for 30 minutes (2, 36). It does not lose biological activity after treatment with pronase, trypsin, proteinase K, deoxyribonuclease, ribonuclease and phospholipase C (2, 48). Acetone, phenol, chloroform and methanol have no effect on the molecule. It is stable to acid even at pH 1 but loses activity at pH greater than 9. Stable toxin can be detected in suckling mice and rabbit ileal loops (30, 36, 68). The onset of fluid accumulation in rabbit ileal loops is rapid with maximum volume being produced between 4-6 hours after inoculation (30). ST does not stimulate adenylate cyclase but appears to activate increased levels of cyclic guanosine 3', 5' monophosphate (GMP) in intestinal tissue by activating guanylate cyclase (56).

Labile toxin has a molecular weight of 73,000 daltons (56). It has two components with molecular weights of 44,000 and 30,000 (56). Its activity is increased by trypsin and completely destroyed by pronase and proteinase K (56). This toxin is labile to heat and acid but is stable to alkaline conditions. Its mechanism of action is similar to cholera toxin (45). LT also appears to be antigenically related to cholera toxin (15, 16, 17). Anti-cholera toxin neutralizes the toxic activity of

cholera toxin as well as labile toxin *in vivo* (15, 16, 17). Cholera toxin also shows immunologic relatedness with LT in immunodiffusion tests. Anti-LT sera produce a precipitin band with both LT and CT and neutralize both toxins. Klipstein et al. reported weak neutralization of stable toxin with anti-LT and anti-CT (53). Labile toxin can be assayed in rabbit ileal loops, skin permeability tests, Y_1 adrenal cells, CHO cells, and pigeon erythrocyte lysates. The suckling mouse in non-responsive (21, 48). Onset of fluid response in rabbit ileal loops is immediate at high doses but delayed at low doses (30). The maximum volume is produced in no less than 10 hours. The ability of the organisms to attach to intestinal epithelium may be a prerequisite for enteric infection. Colonization of the small intestine is followed by enterotoxin production. Intestinal colonization is mediated by pili antigens designated as K 88 in swine (49), K 99 in cows and sheep (47), and colonization factor antigen (CFA) in man (31).

Enteroinvasive *E. coli* have the ability to produce a dysentery like illness similar to that caused by *Shigella*. Invasive *E. coli* invade the epithelial cells of the colon. The organisms cause destruction of the brush border and are engulfed by an envagination of the cell membrane (70). The organisms are contained within vacuoles in epithelial cells from which they invade and kill adjacent cells. This results in inflammation and erosion or ulceration of the epithelium with the production of a dysentery like diseases manifested by bloody mucoid stools.

Salmonella Enterotoxin

Salmonella enterotoxin is less well defined. Gianella studied invasive and noninvasive strains of Salmonella (37). He found that noninvasive strains of Salmonella did not invoke a fluid response while 3 of 6 invasive strains did elicit a positive response. Whole organisms were necessary for fluid accumulation. Sterile filtrates elicited no response. Sedlock, Koupal and Deibel on the other hand reported activity in suckling mice with culture filtrates (81). Sedlock and Deibel reported that responses in rabbit ileal loops could be enhanced if prior to administration of culture supernatant the intestinal lumen is washed with a mucolytic agent. Fluid secretion could also be observed in untreated intestinal loops if enterotoxin was administered with a live, invasive Salmonella strain which did not evoke a secretory response (81). Koupal and Deibel found that Salmonella toxin was heat stable, pH stable, pronase sensitive and shared some properties with E. coli labile and stable toxins (54). Data from our laboratory indicate that live viable organisms are essential for toxic activity. Culture filtrates, concentrates of filtrates as well as sonicates and heat inactivated culture supernatants did not produce activity. It seems that the suckling mouse system is not assaying for a true enterotoxin such as CT or LT with Salmonella. Enterotoxin activity with Salmonella can be assayed in rabbit ileal loops, skin permeability assays and the suckling mouse (54, 77, 80). The exact mechanism of suckling mouse reactivity with Salmonella is not yet understood since Salmonella enterotoxin does not appear to activate adenylate cyclase by the same mechanism as V. cholera toxin (38).

Aeromonas Enterotoxin

Much of the early literature on Aeromonas dealt with classification of the organism or its pathogenesis for fish and frogs (26, 27, 71, 83). Only recently has Aeromonas been considered a possible human pathogen with the ability to produce an enterotoxin (3, 4, 13, 43). Annapurna and Sanyal tested 50 strains of Aeromonas hydrophila isolated from diarrheic and healthy human feces, from domestic animals (cow, buffalo, goat and chickens), from drinking water, sewage and the river Ganges (4). Live cultures and cell-free filtrates of 47 and the 50 strains gave positive responses in rabbit ileal loops. The three negative strains became positive after two passages in ileal loops of rabbits. The amount of fluid produced was comparable to that of a toxigenic strain of Vibrio cholera. The enterotoxin was inactivated after heating at 60°C for 20 minutes and 65° for 10 minutes. The enterotoxin could be precipitated with ammonium sulphate and was non-dialysable. They believed that the toxin was protein. They also could find no histopathologic changes in ileal loops other than the depletion of mucus from goblet cells. Dubey and Sanyal also found that cell-free culture filtrates and crude enterotoxin preparations caused fluid accumulation in rabtit ileal loops (24). The activity was lost after heating at 60° for 20 minutes or 56°C for 30 minutes. The toxin was acid labile and maximum activity was produced at a pH range of 8-10.

Boulanger, Lallier and Cousineau isolated Aeromonas sobria from healthy fish and Aeromonas hydrophila from both healthy and moribund fish (13). Six of the eight Aeromonas sobria strains produced one type of hemolysis on 5% bovine blood agar while 12 of 13 strains of

Aeromonas hydrophila produced two types of hemolysis suggesting that Aeromonas hydrophila was more hemolytic than Aeromonas sobria. They also found that the Aeromonads were proteolytic and produced a cytotoxin. All of the Aeromonas sobria isolates reacted positively in the suckling mouse and rabbit ileal loops. Four of the Aeromonas hydrophila strains were positive in both the suckling mouse and rabbit ileal loop while seven strains were positive in the suckling mouse only. These studies seemed to suggest that Aeromonas might produce two types of toxin; the first, a toxin that produces an immediate response and is detectable in suckling mice and the second a toxin that produces a delayed response and is detectable in rabbit ileal loops. This would indicate that Aeromonas enterotoxin has properties in common with both the stable and labile toxins of E. coli. These investigators could find no antigenic relationship between the toxins from Aeromonas and E. coli.

Sanyal, Singh and Sen compared the enterotoxicity of Aeromonas hydrophila isolated from healthy and diarrheagenic adults and children with isolates of Plesiomonas shigelloides (78). They found that, regardless of source, 12 of 14 strains of Aeromonas hydrophila produced a positive response in rabbit ileal loops while very few of the Plesiomonas shigelloides gave a positive response.

Ljungh, Popoff and Wadström reported isolating an enterotoxin from eleven human isolates of Aeromonas (57). Enterotoxin was assayed in rabbit intestinal loops, rabbit skin and Y_1 adrenal cells. These investigators reported interference in all three assay systems by hemolysins and a cytotoxic protein produced by the Aeromonads. This interference could be inactivated by heating at 56° for 10 minutes or by a specific

antihemolysin. They also found that neither cholera antitoxin nor antiserum to E. coli enterotoxin could neutralize the activity. The action of Aeromonas enterotoxin may be similar to cholera toxin since both cause the release of corticosteroids from Y_1 adrenal cells. These investigators also reported that Plesiomonas shigelloides produced only low levels of enterotoxin.

Gurwith, Bourque, Cameron, Forrest and Green reported isolation of Aeromonas hydrophila from a 67 year old Indian patient with a cholera-like illness (43). Culture filtrates were cytotoxic in mouse adrenal cells, HeLa cells and human foreskin fibroblasts. The toxin was heat and acid labile had an estimated molecular weight of 45,000 daltons. When tested in suckling mice, live cultures and sterile filtrates produced less than positive results but death occurred in all mice in 4 hours. Intraperitoneal injection into adult mice caused death within 24 hours. Fluid accumulation could be demonstrated in rabbit ileal loops.

Cumberbatch et al. studied 96 isolates of Aeromonas hydrophila for cytotoxin and hemolysin production (18). Sixty-six isolates were both cytotoxic and hemolytic while the others produced neither cytotoxin nor hemolysin. Cytotoxic activity correlated with enterotoxic activity as well as with a positive lysine decarboxylase phenotype or a positive Voges-Proskauer phenotype. Enterotoxic activity could not be correlated with the presence or absence of plasmids. They also isolated in low titer a substance that causes rounding and detachment of MAT cells and HeLa cells. They believed that this rounding factor does not resemble the cytotoxic enterotoxins of E. coli and Vibrio cholera, since these enterotoxins have no effect on HeLa cells. These authors believe the cytotoxin

to be a proteolytic substance produced by the Aeromonads. Whether it contributes to the enteropathogenicity of Aeromonas cannot be determined at the present time.

Wadström, Ljungh and Wretlind also found enterotoxin activity in Aeromonas using rabbit ileal loops, rabbit skin tests and Y_1 adrenal cells (89). They noticed that the skin test could be affected by the hemolytic and cytotoxic factors produced by the organism. Heating the test samples at 56° for 10 minutes inactivated the cytotoxic and hemolytic factors. Hemolysins also lysed the adrenal cells but heating inhibited this activity. Addition of specific anti-hemolysins and incubation at 20° for 30 minutes also inhibited the hemolytic activity and allowed detection of enterotoxin in skin tests and adrenal cell tests. Heating of crude enterotoxin samples at 80° for 10 minutes showed that the toxin is heat labile like the enterotoxins of Vibrio cholera and E. coli.

Bernheimer and Avigad have isolated an extracellular hemolytic toxin which they have called "aerolysin" (10). They estimated its molecular weight to be 50,000 daltons. Aerolysin is heat labile and apparently protein. Wretlind, Möllby and Wadström isolated two hemolysins from Aeromonas hydrophila which were cytotoxic for HeLa cells and human fibroblasts and produced dermonecrosis in rabbits (92, 93). These hemolysins were unstable to heating and were inactivated by proteolytic enzymes.

Donta and Haddow also reported cytotoxic activity for Aeromonas hydrophila (23). Their cytotoxin also was heat labile. They could not find enterotoxic activity either by rabbit ileal loop assay or Y_1 adrenal cell assay.

Animal and Cell Models for Detection of Enterotoxin Production

A variety of animal and cell models have been utilized to study enterotoxin activity (21, 29, 32, 42, 79, 82). The rabbit ileal loop was one of the first systems utilized (30, 37, 44). With this procedure, rabbits are fasted for 48 hours prior to use. Under local anesthetic, the small bowel is flushed with phosphate buffered saline and ligated segments averaging 4 to 5 centimeters in length are prepared. Each segment receives a 1.0 ml intraluminal injection of enterotoxic material, PBS (negative control) or cholera toxin (positive control). All samples are injected at least in duplicate and in random sequence. After 18 hours, the ileal sections are excised and measurements are taken of the volume of fluid accumulated with the segment.

Another animal model that has been utilized is the suckling mouse (50, 62, 66). Dean et al. developed the system to detect ST production by *E. coli* (21). The mice used in the assay were 1-4 days old. He injected the test material through the body wall directly into the milk-filled stomachs of the mice. After 4 hours, the mice were sacrificed, the intestines removed and examined for fluid accumulation. Giannella modified the assay by suggesting the incubation time be changed to 3 hours, the growth medium for the bacteria be Casamino acids--yeast extract broth, and that roller cultures be used to obtain better toxin production (36). The assay was further investigated by Moon et al. who reported optimal results when mice used in the assay were less than 6 days old (62). False negatives were produced if incubation was performed at 37°C and older mice were used.

The rabbit skin permeability assay was another system developed to detect enterotoxic activity (29, 32). Culture filtrates of enterotoxic organisms produce erythema, induration and increased capillary permeability when injected into the skin of rabbits. Injection of Evan's blue dye several hours after the culture filtrates enhanced the reading of the test. A rapid blanching reaction was observed with E. coli and Salmonella if the dye was injected 1 hour after the filtrates. This factor was heat stable in contrast to delayed permeability factor which was heat labile (77).

In vitro cell systems such as Y_1 adrenal cells and Chinese hamster ovary cells (CHO) have also been frequently used (22, 41, 43, 86). In the Y_1 assay, enterotoxic activity is detected by morphological rounding and steroidogenesis in the monolayer cultures. The CHO cells respond by activation of adenylate cyclase with accumulation of cAmp and cell elongation. CHO cells are 100 to 10,000 times more sensitive for cholera toxin and 5 to 100 times more sensitive for LT than either the rabbit ileal loop or the rabbit skin permeability tests.

MATERIALS AND METHODS

Bacterial Strains

Aeromonas hydrophila strains Em-56, E-3, E-16, E-14 and E-2 were of environmental origins. Strain Cl-7 was isolated from a diver leg wound. *Aeromonas sobria* strains Cl-124 and Cl-125 were isolated from blood cultures while strain 54 was of environmental origin. All strain were kindly provided by Dr. Richard Walker of the Naval Medical Research Command, Bethesda, MD. *Escherichia coli* H10407 was obtained from J. W. Peterson, University of Texas, Galveston. *Salmonella typhimurium* E9288 was obtained from E. V. Morse, School of Veterinary Medicine, Purdue University, Lafayette, Indiana.

Media and Maintenance of Cultures

All cultures were maintained on agar slants consisting of 2.0% peptone (Difco Laboratories, Detroit, Michigan) 0.5% NaCl and 2.0% agar (32) at 4° with transfers made every 6-10 weeks. For permanent storage, all strains were cultured overnight in 10 ml of Brain Heart Infusion (BHI) broth (Difco Laboratories). The cultures were centrifuged at 12,100 x g for 20 minutes in a Sorvall RC-5 Superspeed Refrigerated Centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut). The bacterial cell pellets were washed two times with 0.85% NaCl. Five ml of glycerol (Mallinckrodt, St. Louis, Missouri) were added and the suspension was maintained at -20°C.

Preparation of Cultural Supernatants

Organisms were inoculated from agar slants into 5 ml of BHI broth in 150 x 16 mm tubes. The tubes were placed in a Wheaton Roller Culture Apparatus (Wheaton Scientific, Millville, New Jersey), set at ½ rev/min and incubated at 37°C for 18-24 hours. The cultures were then centrifuged in a Sorvall RC-5 Superspeed Refrigerated Centrifuge at 12,100 x g for 20 minutes. The resultant broth supernatants were used to inoculate suckling mice, except where filtration is specified.

Filtration and Concentration

Culture supernatants were filtered through 0.45 μm Swinnex 13 membrane filters (Millipore Corporation, Bedford, Massachusetts). The filtrates were assayed for enterotoxic activity. Filtrates were concentrated ten-fold with an Amicon ultrafiltration unit fitted with a PM 30 membrane (Amicon Corporation, Lexington, Massachusetts). Retentates and filtrates were both tested in suckling mice.

Mice

Newborn HA/ICR suckling mice (Harlan Industries, Incorporated, Cumberland, Indiana) 3 to 5 days old were used in the suckling mouse assay. Mice were separated from their mothers immediately before use. The average of three mice was used to determine each experimental point.

Suckling Mouse Assay

The procedures of Dean et al. were performed with the following modification. Each of three mice were injected intragastrically (percutaneously with a 30½ gauge needle) with either 0.1 ml of culture supernatant, filtrate or concentrate containing two drops of 2% Evans blue dye/ml. The mice were incubated for 2½ hours at room temperature and then sacrificed by cervical dislocation. The abdomen was opened and the entire intestine, excluding the stomach was removed with forceps and weighed. The ratio of gut weight to remaining carcass weight was calculated and an average value obtained for each group of 3 mice. Activity is expressed as the ratio of intestinal weight to remaining body weight (IW/BW). Values greater than or equal to .086 are considered positive, values between .080 and .085 are indeterminate and values less than or equal to .079 are negative. Each assay was performed at least in duplicate utilizing three mice per assay.

Hemagglutination Assay

Hemagglutination tests were performed according to the methods of Deguid et al. (25) with the following alterations. Citrated guinea pig, sheep or human red blood cells were washed three times in saline and made up to a 3% suspension in saline. Alternatively, red cells were washed and resuspended in saline containing 0.5% mannose to test for susceptibility of hemagglutination to mannose. Sedimented bacterial cells from BHI roller tubes, tryptose slants, peptone slants, 1% peptone roller tubes and human blood agar plates were suspended in 0.25 ml saline.

Two drops of bacterial suspension were tested with 2 drops of red cell suspension with or without mannose.

Column Chromatography

Following ultrafiltration, selected positive filtrates were chromatographed on a 25 x 2.6 cm. G-100 Sephadex column (Pharmacia Fine Chemicals Inc., Piscataway, New Jersey) equilibrated with 0.1 N tris-HCl buffer at pH 7.0. Chromatography was carried out at 5°C. One ml fractions were collected. Protein determinations were carried out on these fractions by the method of Lowry (59). Fractions were pooled into groups of 10 and tested in suckling mice.

Statistics

The mean of IW/BW ratios ± 1 standard error of the mean for each sample tested was calculated. The student's t-test was used to evaluate results when indicated (11).

RESULTS

Enterotoxic Activity in Culture Supernatants

Culture supernatants from all strains of Aeromonas sobria showed positive activity in the suckling mouse (IW/BW $\geq$ .086). Of those, two were of human origin (C1-124 and C1-125 both isolated from blood cultures) and one (strain 54) of environmental origin. Positive responses were also seen with two strains of Aeromonas hydrophila (Em-56 of environmental origin and C1-7 isolated from a diver's leg wound). Three strains of Aeromonas hydrophila (E-16, E-14 and E-2) were negative (IW/BW $\leq$ .079). All three were of environmental origin. Strain E-3 of Aeromonas hydrophila was indeterminate in the suckling mouse. All the Aeromonads were found to be motile indicating that motility does not seem to correlate with enterotoxic activity (Table 2).

Mannose Sensitive Hemagglutinating Activity of Aeromonas Organisms

Organisms were grown in various media (BHI broth, tryptose slants, 1% peptone broth and slants, and on human blood agar plates) and tested for hemagglutinating activity with guinea pig red cells, sheep red cells and human O red cells with and without mannose. Only strains 54, E-16 and E-14 possessed mannose sensitive hemagglutinating activity with

Table 1. *Aeromonas* Species and Source of Isolation

Number	Species	Source
Cl-124	*Aeromonas sobria*	Blood culture
Em-56	*Aeromonas hydrophila*	Environmental
Cl-125	*Aeromonas sobria*	Blood culture
54	*Aeromonas sobria*	Environmental
Cl-7	*Aeromonas hydrophila*	Diver's leg wound
E-3	*Aeromonas hydrophila*	Environmental
E-16	*Aeromonas hydrophila*	Environmental
E-14	*Aeromonas hydrophila*	Environmental
E-2	*Aeromonas hydrophila*	Environmental

Table 2. Production of Enterotoxin-like Activity Assayed by the Suckling Mouse Model in BHI Roller Tube Culture Supernatants of *Aeromonas*[a]

Number	IW/BW Ratio	Organism	Motility
C1-124	.103 ± .008 (9)	*Aeromonas sobria*	+
Em-56	.102 ± .008 (11)	*Aeromonas hydrophila*	+
C1-125	.097 ± .004 (11)	*Aeromonas sobria*	+
54	.090 ± .002 (10)	*Aeromonas sobria*	+
C1-7	.086 ± .003 (9)	*Aeromonas hydrophila*	+
E-3	.085 ± .002 (8)	*Aeromonas hydrophila*	+
E-16	.079 ± .005 (9)	*Aeromonas hydrophila*	+
E-14	.077 ± .005 (8)	*Aeromonas hydrophila*	+
E-2	.074 ± .003 (6)	*Aeromonas hydrophila*	+
E-9288	.105 ± .004 (9)	*Salmonella typhimurium*	ND
H10407	.130 ± .015 (6)	*Escherichia coli*	ND
BHI broth	.075 ± .003 (7)		

[a]Figures represent mean ± 1 standard error of the mean.
Numbers in parentheses indicate the number of separate tests performed.

guinea pig red cells (Table 3). Growth on slants or in roller tube cultures did not affect the hemagglutinating ability of the strains.

Enterotoxic Response in Suckling Mice with Aeromonas spp. Following Filter Sterilization and Ultrafiltration

To test for the presence of a cell-free exotoxin, culture supernatants were filter sterilized through a 0.45 micron milipore filter. Results in Table 4 show that enterotoxic activity was not lost by passage through a filter. The five positive strains of Aeromonas remained positive, the three negative strains remained negative and the indeterminate strain remained indeterminate.

The possibility that small amounts of enterotoxin were being produced by the negative strains was also investigated. Culture filtrates were concentrated ten-fold by ultrafiltration with a PM 30 membrane. The retentates and filtrates from the Amicon were tested in suckling mice. Results in Table 5 show that ten-fold concentration did not increase suckling mouse reactivity.

When the positive strains were concentrated ten-fold, enterotoxic activity could only be found in the retentate. This suggests that the toxic molecule is of 30,000 or greater molecular weight. Uninoculated broth concentrated ten-fold was negative.

Enterotoxic Activity Following Heating of Culture Filtrates

Culture filtrates of Aeromonas sobria (Cl-124, Cl-125 and 54) and Aeromonas hydrophila (Em-56) were heated in a 60° waterbath for 10, 15,

Table 3. Hemagglutinating Ability of *Aeromonas* Isolates

Number		Guinea Pig RBC's	Sheep and Human RBC's	Enterotoxicity
C1-124	BHI broth	-	-	+
	other media[a]	-	-	ND
Em-56	BHI broth	-	-	+
	other media	-	-	ND
C1-125	BHI broth	-	-	+
	other media	-	-	ND
54[b]	BHI broth	+	-	+
	other media	+	-	ND
C1-7	BHI broth	-	-	+
	other media	-	-	ND
E-3	BHI broth	-	-	indeterm
	other media	-	-	ND
E-16[b]	BHI broth	+	-	-
	other media	+	-	ND
E-14[b]	BHI broth	+	-	-
	other media	+	-	ND

[a]Other media include BHI broth, tryptose slants, human blood agar plates, 1% peptone broth and slants.

[b]Cultures were positive when tested with guinea pig red cells and grown on all media except 1% peptone broth and slants.

Table 4. Effect of Filter Sterilization on the Enterotoxic Activity of *Aeromonas*[a]

Number	Unfiltered IW/BW Ratio	Filtered IW/BW Ratio
C1-124	.103 ± .008 (9)	.104 ± .004 (7)
Em-56	.102 ± .008 (11)	.096 ± .008 (8)
C1-125	.097 ± .004 (11)	.103 ± .006 (7)
54	.090 ± .002 (10)	.091 ± .005 (5)
C1-7	.086 ± .003 (9)	.084 ± .002 (4)
E-3	.085 ± .002 (8)	.083 ± .002 (3)
E-16	.079 ± .005 (9)	.075 ± .004 (3)
E-14	.077 ± .005 (8)	.080 ± .007 (3)
E-2	.074 ± .003 (6)	.073 ± .001 (5)
E-9288 (Salmonella)	.105 ± .004 (9)	.076 ± .007 (6)

[a]Figures represent mean ± 1 standard error of the mean.
Numbers in parentheses indicate the number of separate tests performed.

Table 5. Reactivity of Filtrates Concentrated Ten-fold by Ultrafiltration with a PM 30 Membrane Filter

Number	IW/BW[a]			
	Culture Supernatant	Filtrate	10X Concentrate	Filtrate (Amicon)
Cl-124	.103 ± .008 (9)	.104 ± .004 (7)	.092 ± .005 (5)	.078 ± .002 (3)
Em-56	.102 ± .008 (11)	.096 ± .008 (8)	.102 ± .004 (2)	.082 ± .002 (3)
Cl-125	.097 ± .004 (11)	.103 ± .006 (7)	.094 ± .002 (3)	.076 ± .001 (3)
54	.090 ± .002 (10)	.091 ± .005 (5)	.092 ± .010 (3)	.073 ± .004 (3)
Cl-7	.086 ± .003 (9)	.084 ± .002 (4)	.086 ± .000 (3)	.078 ± .002 (3)
E-3	.085 ± .002 (8)	.083 ± .002 (3)	.080 ± .003 (3)	.079 ± .001 (3)
E-16	.079 ± .005 (9)	.075 ± .004 (3)	.079 ± .003 (3)	.077 ± .004 (3)
E-14	.077 ± .005 (8)	.080 ± .007 (3)	.077 ± .001 (3)	.079 ± .001 (2)
E-2	.074 ± .003 (6)	.073 ± .001 (5)	.074 ± .002 (3)	.075 ± .003 (3)
Broth	.075 ± .003 (7)	-	.084 ± .003 (3)	-

[a]Figures represent mean ± 1 standard error of the mean.
Numbers in parentheses indicate the number of separate tests performed.

and 20 minutes respectively, cooled to room temperature and injected into suckling mice. Table 6 shows a gradual loss of enterotoxic activity with prolonged heat treatment. E. coli enterotoxin treated in a similar fashion did not show a loss of activity. Heating for 20 minutes or longer destroys all enterotoxic activity in Aeromonads.

Passage of Concentrate Through G-100 Sephadex Column

Culture filtrates of strains Em-56, Cl-124 and broth were concentrated ten-fold with an Amicon PM 30. Two mls of this concentrate was layered onto a Sephadex G-100 column. One ml fractions were collected. These fractions were tested for protein content by the method of Lowry and then pooled in groups of 10 and tested in suckling mice. Figures 1, 2, and 3 represent the graphs of protein concentration of each of the fractions. Enterotoxic activity is represented by bars. The greatest protein concentration can be found in tubes 40-50 of both the broth and culture fractions. Enterotoxic activity can be seen in pooled fractions 11-19 and 20-29 for the Aeromonas strains. No enterotoxic activity could be detected in the fractions of sterile broth. The large concentration of protein in tubes 40-50 can best be explained as unused constituents of the BHI broth.

Table 6. Effect of Heating at 60°C on Enterotoxic Activity of Aeromonas

Number	IW/BW[a]			
	Culture Supernatant	60° - 10 min	60° - 15 min	60° - 20 min
Cl-124	.103 ± .008 (9)	.090 ± .003 (3)	.004 ± .002 (3)	.078 ± .003 (4)
Em-56	.102 ± .008 (11)	.089 ± .006 (3)	.089 ± .003 (2)	.076 ± .000 (5)
Cl-125	.097 ± .004 (11)	.094 ± .000 (2)	.084 ± .001 (2)	.075 ± .003 (5)
54	.090 ± .002 (10)	.090 ± .004 (2)	.079 ± .005 (3)	.076 ± .002 (5)

[a]Figures represent mean ± 1 standard error of the mean.
Numbers in parentheses indicate the number of separate tests performed.

Figure 1. Enterotoxin activity (bars) and protein concentration (line) of filtrates from Aeromonas strain Em-56 following Sephadex G-100 chromatography. Numbers in parentheses indicate the number of separate tests performed.

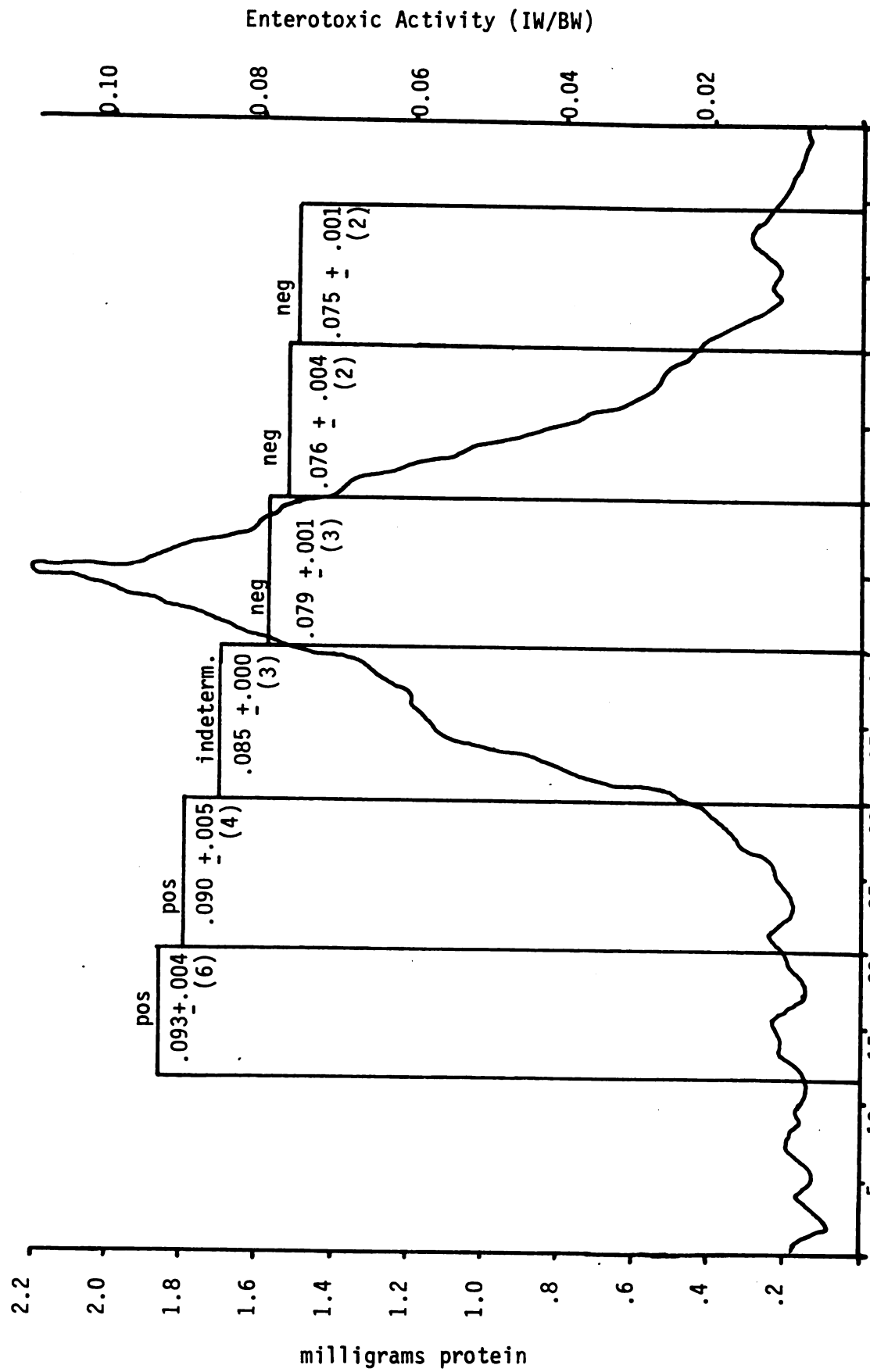
Enterotoxic Activity (IW/BW)
0.10
0.08
0.06
0.04
0.02
pos
.093±.004
(6)
pos
.090 ±.005
(4)
indeterm.
.085 ±.000
(3)
neg
.079 ±.001
(3)
neg
.076 ± .004
(2)
neg
.075 ± .001
(2)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
milligrams protein
5
10
15
20
25
30
35
40
45
50
55
60
65
70
Tube Number

Figure 1

Figure 2. Enterotoxin activity (bars) and protein concentration (line) of filtrates from Aeromonas strain C1-124 following Sephadex G-100 chromatography. Number in parentheses indicate the number of separate tests performed.

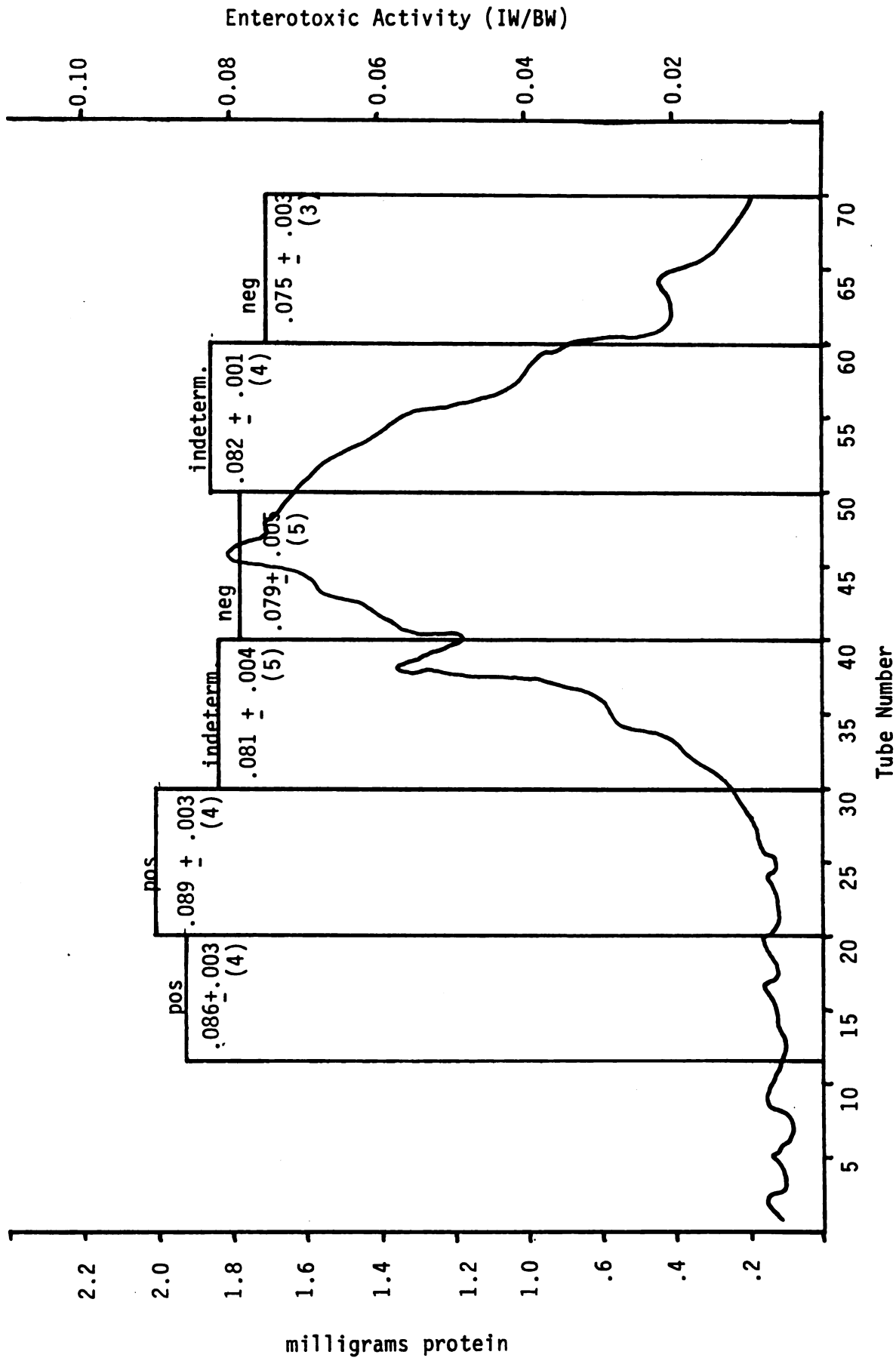
Enterotoxic Activity (IW/BW)
0.10
0.08
0.06
0.04
0.02
pos
.086+.003
(4)
pos
.089 ± .003
(4)
indeterm.
.081 ± .004
(5)
neg
.079+.005
(5)
indeterm.
.082 ± .001
(4)
neg
.075 ± .003
(3)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.6
.4
.2
milligrams protein
5
10
15
20
25
30
35
40
45
50
55
60
65
70
Tube Number

Figure 2

Figure 3. Enterotoxin activity (bars) and protein concentration (line) of concentrated BHI broth following Sephadex G-100 chromatography. Numbers in parentheses indicate the number of separate tests performed.

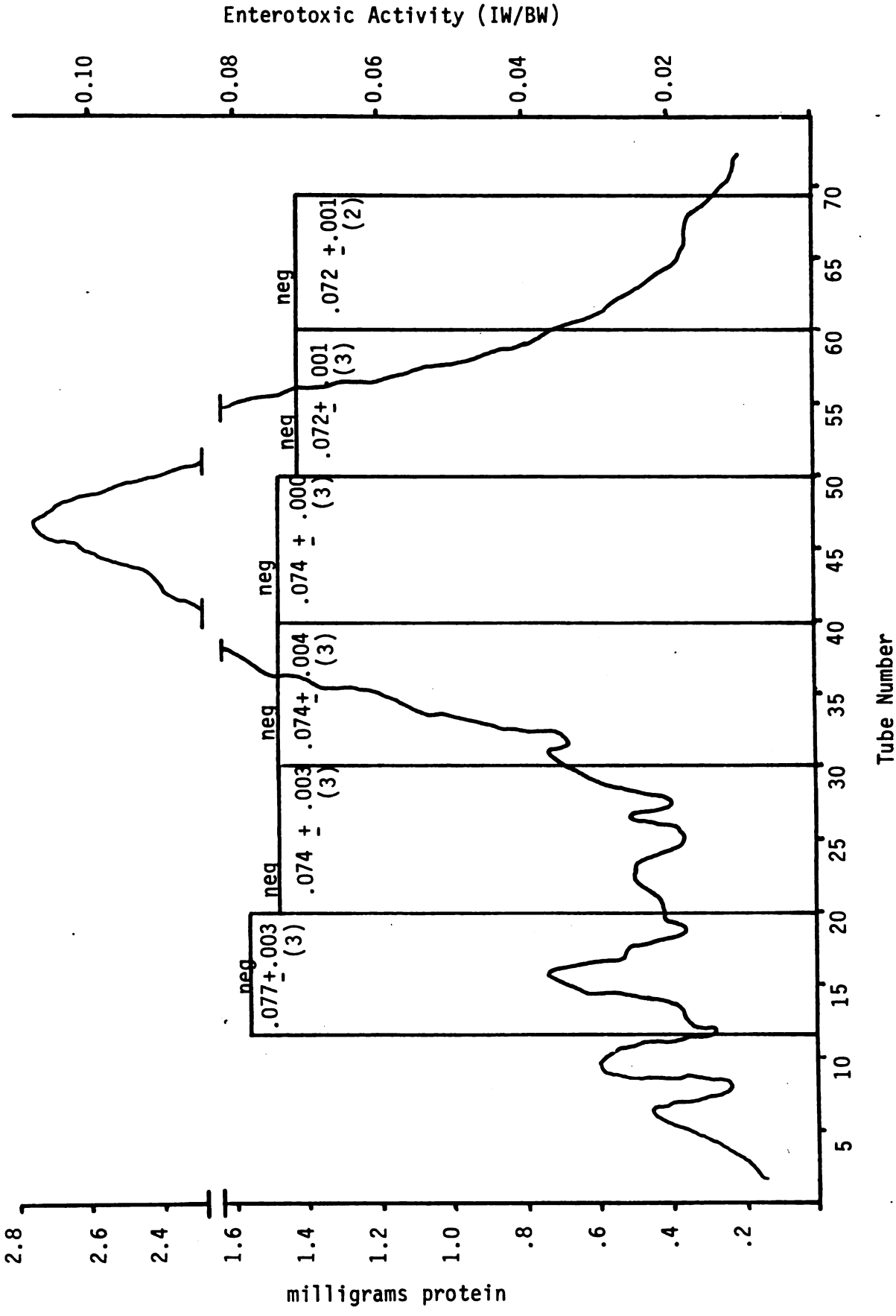
Enterotoxic Activity (IW/BW)
0.10
0.08
0.06
0.04
0.02
neg
.077+.003 (3)
neg
.074 ± .003 (3)
neg
.074+.004 (3)
neg
.074 ± .000 (3)
neg
.072+.001 (3)
neg
.072 ±.001 (2)
2.8
2.6
2.4
1.6
1.4
1.2
1.0
.8
.6
.4
.2
milligrams protein
5
10
15
20
25
30
35
40
45
50
55
60
65
70
Tube Number

Figure 3

DISCUSSION

Many organisms previously considered to be nonpathogenic for humans have recently been implicated in diarrhea outbreaks. These same organisms cause fluid accumulation in the rabbit ileal loops (8, 43). Among these, Aeromonas spp. have been associated with diarrhea and many investigators have studied the enterotoxic potential of organisms in this genus (3, 4, 43). Several studies have examined the enterotoxic activity of Aeromonas in rabbit ileal loops, the rabbit skin test, the adrenal cell assay and the suckling mouse (3, 4, 13, 24, 43, 71). In this study, 5 of the 9 strains obtained from researchers at the Naval Medical Research Institute were shown to be enterotoxic in the suckling mouse assay while 1 was indeterminate and 3 were negative. All of the strains isolated from humans were positive.

True enterotoxins are exotoxins which are released into the culture medium during growth of an organism. Cell-free preparations of cultures of E. coli and V. cholera can cause fluid accumulation in both rabbit ileal loops and suckling mice (7, 8, 21, 64). Salmonella, according to Gianella, only induce a positive response when whole organisms are present (37). Yet, Sedlock, Koupal and Deibel report activity in suckling mice with culture filtrates (81). Annapurna, Sanyal and other investigators have reported positive responses in suckling mice and rabbit ileal loop assays with cell-free preparations of Aeromonas cultures (3, 4, 24, 57, 79). In this study, culture supernatants which were

filter sterilized by passage through a 0.45 milipore filter still retained the ability to induce a positive response in suckling mice. It appears, therefore, that the Aeromonas enterotoxin, like the E. coli and Virbrio cholera enterotoxin, is a cell-free exotoxin, released into the medium by the growing organism.

Since some investigators found that toxic material can be retained by a PM 30 membrane, positive strains were concentrated in the Amicon unit (77, 81). In this study, only the retentates from the PM 30 showed positive activity. Filtrates from the PM 30 were negative in suckling mice. This would indicate that the toxin has a molecular weight of 30,000 or greater. Since the Aeromonads showing a negative or indeterminate response might be producing toxin at a low level, attempts were also made to concentrate the filtrates with an Amicon PM 30 ultrafiltration unit. After ten-fold concentration, no increase in suckling mouse reactivity was found.

Annapurna and Sanyal have reported loss of enterotoxic activity in rabbit ileal loops of Aeromonas culture filtrates after heating (3, 4). It was desired to know if this phenomenon could also be seen in the suckling mouse assay. A gradual loss of enterotoxic activity could be seen with prolonged heat treatment. No enterotoxic activity remained after heating culture filtrates for 20 minutes at 60°C. This indicates that Aeromonas enterotoxin may be similar to E. coli labile toxin and Vibrio cholera enterotoxin.

Since it was believed that the enterotoxic molecule may be protein in nature, column chromatography was performed with concominant protein analysis of the resulting fractions. Although individual peaks of

protein could not be isolated (possibly due to the protein rich BHI broth used for growth of the Aeromonads), enterotoxic activity could be found only in 2 pools of the fractions. Since these pools were eluted very early from the column, it can be postulated that the toxin is a relatively large molecule with a molecular weight ranging from 30,000 to 150,000 daltons. It is not clear at this point whether the protein present in these fractions is due to the enterotoxin or to constituents of the growth media.

For enterotoxigenic organisms to be pathogenic, the organisms must first be able to adhere to the intestinal mucosa. Many investigators have studied properties of the organisms that might be responsible for adherence. With V. cholera, adhesion has been associated with motility (35). Studies performed with the Aeromonads (see Table 2) showed that all the isolates, including those strains negative in the suckling mouse, were motile indicating that the same mechanism might not be operating here. In enterotoxigenic E. coli, adherence is determined by the presence of K antigens in animals and colonization factor antigens in humans. These antigens were non-flagellar, surface appendages believed to be pili. If these antigens are lost, the organisms lose their ability to produce disease even though they still retain their enterotoxigenicity (61). Coliform bacteria also have the ability to agglutinate red blood cells and this property has been attributed to the presence of pili (76). Hemagglutination attributed to Type 1 pili can be inhibited by low concentrations of mannose (5). Studies were undertaken to determine if piliation, the ability to attach to buccal epithelial cells or the hemagglutinating ability of the Aeromonads had any bearing on

enterotoxic activity and disease production. Transmission electron microscopy studies performed at the Naval Medical Research Institute showed that all of the Aeromonads possessed pili. The Aeromonads also showed variability in regard to attachment to buccal epithelial cells (see Appendix 1). From these studies, no correlation could be established for these parameters with enterotoxic activity or disease production. Hemagglutination studies performed in our laboratory with guinea pig red cells showed that three strains possessed mannose sensitive hemagglutinating pili, but that only one of these strains was enterotoxic. Since some investigators believe that pili production is enhanced with the use of enriched media (human blood agar, peptone broth, tryptose agar), further agglutination studies were performed. The bacteria were grown in BHI broth, 1% peptone slants and broth, tryptose slants and on human blood agar plates. In addition, human group O red cells and sheep red blood cells both with and without mannose were employed. Variations in temperature were also tested (data not shown). In each case, only the 3 strains that showed hemagglutinating ability with the guinea pig red cells showed hemagglutinating ability under variations in growth conditions. Agglutination was seen only with guinea pig red cells. Sheep and human O cells did not agglutinate. Possession of hemagglutinating pili appears not to be correlated with enterotoxin production.

Some investigators report a correlation of enterotoxic activity with the production of a cytotoxin (18, 89). In this study, all strains that produced an enterotoxin were also cytotoxic for Y_1 adrenal cells (see Appendix 1). It is not clear at the present time if this represents the same toxic activity. Suckling mouse reactivity was also correlated with a lower LD_{50} for adult mice.

SUMMARY

Aeromonads, like Vibrios, cause little change in the gastric epithelium when producing disease indicating that a toxin is responsible for disease production. From these studies, it can be concluded that Aeromonads have the ability to produce an enterotoxin. The toxin, like V. cholera toxin, is filterable and heat labile. Both toxins can be assayed in the same animal models. Like cholera toxin, Aeromonas toxin induces steroidogenesis in Y_1 adrenal cells. Enterotoxic activity in Aeromonads is correlated with cytotoxic activity and a low LD_{50}. Motility, the possession of hemagglutinating pili and the ability to adhere to buccal epithelial cells do not seem to play a role in enterotoxic potential. Cholera toxin is a large molecular weight protein. Aeromonas toxin also appears to be of a relatively large molecular weight (30,000-150,000 daltons). The exact chemical composition is not known.

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APPENDIX

Further tests on the strains performed by researchers at the Naval Medical Research Institute yielded the following data. Piliation studies, performed with transmission electron microscopy, showed that all the strains possessed pili with densities from +/- to 4+. Cytotoxicity studies done using Y_1 adrenal cells indicate that 6 of the 9 isolates produced a cytotoxin. Attachment studies of Aeromonads to buccal epithelial cells produced variable results. LD_{50} studies performed in adult mice showed great variation among the strains. Suckling mouse assays performed in our laboratory detected enterotoxic activity with the strains of Aeromonas which were cytotoxic and had a low LD_{50} for adult mice (Appendix 1). Like the enterotoxic activity, cytotoxic activity was heat labile (data not shown). Apparently, piliation and attachment do not seem to play a role in enterotoxin production. The exact role of cytotoxic potential with regard to enterotoxicity has not been established.

Appendix 1. Comparison of Enterotoxic Activity with Other Properties of the Genus Aeromonas

Number	Enterotoxic Response[a]	Cytotox.[b]	LD_{50}[b]	Attachment[b]	Piliation
Cl-124	positive	+	3.7	5.1	3+
Em-56	positive	+	6.5	1.6	1+
Cl-125	positive	+	6.6	7.2	2+
54	positive	+	4.5	1.2	1+
Cl-7	positive	+	4.5	10.2	4+
E-3	indeterminate	+	5.4	4.1	4+
E-16	negative	-	8.2	1.2	+/-
E-14	negative	-	7.8	1.1	1+
E-2	negative	-	8.4	4.5	1+

[a]Data obtained at Michigan State University, East Lansing, Michigan.

[b]Data obtained at the Naval Medical Research Institute, Bethesda, Md.